

Chittaranjan Kole *Editor*

Wild Crop Relatives: Genomic and Breeding Resources

Legume Crops and Forages

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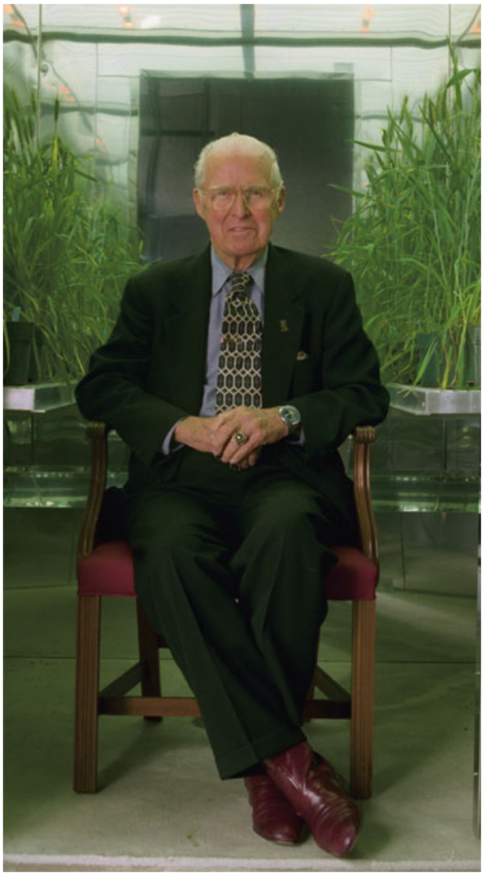
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Dedication



Dr. Norman Ernest Borlaug,[1] the Father of Green Revolution, is well respected for his contributions to science and society. There was or is not and never will be a single person on this Earth whose single-handed service to science could save millions of people from death due to starvation over a period of over four decades like Dr. Borlaug's. Even the Nobel Peace Prize he received in 1970 does not do such a great and noble person as Dr. Borlaug justice. His life and contributions are well known and will remain in the pages of history of science. I wish here only to share some facets of this elegant and ideal personality I had been blessed to observe during my personal interactions with him.

It was early 2007 while I was at the Clemson University as a visiting scientist one of my lab colleagues told me that "somebody wants to talk to you; he appears to be an old man." I took the telephone receiver casually and said hello. The response from the other side was – "I am Norman Borlaug; am I talking to Chitta?" Even a million words would be insufficient to define and depict the exact feelings and thrills I experienced at that moment!

[1]The photo of Dr. Borlaug was kindly provided by Julie Borlaug (Norman Borlaug Institute for International Agriculture, Texas A&M Agriculture) the granddaughter of Dr. Borlaug.

I had seen Dr. Borlaug only once, way back in 1983, when he came to New Delhi, India to deliver the Coromandal Lecture organized by Prof. M.S. Swaminathan on the occasion of the 15th International Genetic Congress. However, my real interaction with him began in 2004 when I had been formulating a 7-volume book series entitled *Genome Mapping and Molecular Breeding in Plants*. Initially, I was neither confident of my ability as a series/book editor nor of the quality of the contents of the book volumes. I sent an email to Dr. Borlaug attaching the table of contents and the tentative outline of the chapters along with manuscripts of only a few sample chapters, including one authored by me and others, to learn about his views as a source of inspiration (or caution!) I was almost sure that a person of his stature would have no time and purpose to get back to a small science worker like me. To my utter (and pleasant) surprise I received an email from him that read: "May all Ph.D.'s, future scientists, and students that are devoted to agriculture get an inspiration as it refers to your work or future work from the pages of this important book. My wholehearted wishes for a success on your important job." I got a shot in my arm (and in mind for sure)! Rest is a pleasant experience – the seven volumes were published by Springer in 2006 and 2007, and were welcome and liked by students, scientists and their societies, libraries, and industries. As a token of my humble regards and gratitude, I sent Dr. Borlaug the Volume I on *Cereals and Millets* that was published in 2006. And here started my discovery of the simplest person on Earth who solved the most complex and critical problem of people on it – hunger and death.

Just one month after receiving the volume, Dr. Borlaug called me one day and said, "Chitta, you know I cannot read a lot now-a-days, but I have gone through only on the chapters on wheat, maize and rice. Please excuse me. Other chapters of this and other volumes of the series will be equally excellent, I believe." He was highly excited to know that many other Nobel Laureates including Profs. Arthur Kornberg, Werner Arber, Phillip Sharp, Günter Blobel, and Lee Hartwell also expressed generous comments regarding the utility and impact of the book series on science and the academic society. While we were discussing many other textbooks and review book series that I was editing at that time, again in my night hours for the benefit of students, scientists, and industries, he became emotional and said to me, "Chitta, forget about your original contributions to basic and applied sciences, you deserved Nobel Prize for Peace like me for providing academic foods to millions of starving students and scientists over the world particularly in the developing countries. I will recommend your name for the World Food Prize, but it will not do enough justice to the sacrifice you are doing for science and society in your sleepless nights over so many years. Take some rest Chitta and give time to Phullara, Sourav and Devleena" (he was so particular to ask about my wife and our kids during most of our conversations). I felt honored but really very ashamed as I am aware of my almost insignificant contribution in comparison to his monumental contribution and thousands of scientists over the world are doing at least hundred-times better jobs than me as scientist or author/editor of books! So, I was unable to utter any words for a couple of minutes but realized later that he must been too affectionate to me and his huge affection is the best award for a small science worker as me!

In another occasion he wanted some documents from me. I told him that I will send them as attachments in emails. Immediately he shouted and told me: "You know, Julie (his granddaughter) is not at home now and I cannot check email myself. Julie does this for me. I can type myself in type writer but I am not good in computer. You know what, I have a xerox machine and it receives fax also. Send me

the documents by fax." Here was the ever-present child in him. Julie emailed me later to send the documents as attachment to her as the "xerox machine" of Dr. Borlaug ran out of ink!

Another occasion is when I was talking with him in a low voice, and he immediately chided me: "You know that I cannot hear well now-a-days; I don't know where Julie has kept the hearing apparatus, can't you speak louder?" Here was the fatherly figure who was eager to hear each of my words!

I still shed tears when I remember during one of our telephone conversations he asked: "You know I have never seen you, can you come to Dallas in the near future by chance?" I remember we were going through a financial paucity at that time and I could not make a visit to Dallas (Texas) to see him, though it would have been a great honor.

In late 2007, whenever I tried to talk to Dr. Borlaug, he used to beckon Julie to bring the telephone to him, and in course of time Julie used to keep alive all the communications between us when he slowly succumbed to his health problems.

The remaining volumes of the *Genome Mapping and Molecular Breeding in Plants* series were published in 2007, and I sent him all the seven volumes. I wished to learn about his views. During this period he could not speak and write well. Julie prepared a letter based on his words to her that read: "Dear Chitta, I have reviewed the seven volumes of the series on *Genome Mapping and Molecular Breeding in Plants*, which you have authored. You have brought together genetic linkage maps based on molecular markers for the most important crop species that will be a valuable guide and tool to further molecular crop improvements. Congratulations for a job well done."

During one of our conversations in mid-2007, he asked me what other book projects I was planning for Ph.D. students and scientists (who had always been his all-time beloved folks). I told him that the wealth of wild species already utilized and to be utilized for genetic analysis and improvement of domesticated crop species have not been deliberated in any book project. He was very excited and told me to take up the book project as soon as possible. But during that period I had a huge commitment to editing a number of book volumes and could not start the series he was so interested about.

His sudden demise in September 2009 kept me so morose for a number of months that I could not even communicate my personal loss to Julie. But in the meantime, I formulated a 10-volume series on *Wild Crop Relatives: Genomic and Breeding Resources* for Springer. And whom else to dedicate this series to other than Dr. Borlaug!

I wrote to Julie for her formal permission and she immediately wrote me: "Chitta, Thank you for contacting me and yes I think my grandfather would be honored with the dedication of the series. I remember him talking of you and this undertaking quite often. Congratulations on all that you have accomplished!" This helped me a lot as I could at least feel consoled that I could do a job he wanted me to do and I will always remain grateful to Julie for this help and also for taking care of Dr. Borlaug, not only as his granddaughter but also as the representative of millions of poor people from around the world and hundreds of plant and agricultural scientists who try to follow his philosophy and worship him as a father figure.

It is another sad experience of growing older in life that we walk alone and miss the affectionate shadows, inspirations, encouragements, and blessings from the fatherly figures in our professional and personal lives. How I wish I could treat my next generations in the same way as personalities like Mother Teresa and Dr. Norman Borlaug and many other great people from around the world treated me!

During most of our conversations he used to emphasize on the immediate impact of research on the society and its people. A couple of times he even told me that my works on molecular genetics and biotechnology, particularly of 1980s and 1990s, have high fundamental importance, but I should also do some works that will benefit people immediately. This advice elicited a change in my thoughts and workplans and since then I have been devotedly endeavoring to develop crop varieties enriched with phytomedicines and nutraceuticals. Borlaug influenced both my personal and professional life, particularly my approach to science, and I dedicate this series to him in remembrance of his great contribution to science and society and for all his personal affection, love and blessings for me.

I emailed the above draft of the dedication page to Julie for her views and I wish to complete my humble dedication with great satisfaction with the words of Julie who served as the living ladder for me to reach and stay closer to such as great human being as Dr. Borlaug and express my deep regards and gratitude to her. Julie's email read: "Chitta, Thank you for sending me the draft dedication page. I really enjoyed reading it and I think you captured my grandfather's spirit wonderfully. . .. So thank you very much for your beautiful words. I know he would be and is honored."

Clemson, USA Chittaranjan Kole

Preface

Wild crop relatives have been playing enormously important roles both in the depiction of plant genomes and the genetic improvement of their cultivated counterparts. They have contributed immensely to resolving several fundamental questions, particularly those related to the origin, evolution, phylogenetic relationship, cytological status and inheritance of genes of an array of crop plants; provided several desirable donor genes for the genetic improvement of their domesticated counterparts; and facilitated the innovation of many novel concepts and technologies while working on them directly or while using their resources. More recently, they have even been used for the verification of their potential threats of gene flow from genetically modified plants and invasive habits. Above all, some of them are contributing enormously as model plant species to the elucidation and amelioration of the genomes of crop plant species.

As a matter of fact, as a student, a teacher, and a humble science worker I was, still am and surely will remain fascinated by the wild allies of crop plants for their invaluable wealth for genetics, genomics and breeding in crop plants and as such share a deep concern for their conservation and comprehensive characterization for future utilization. It is by now a well established fact that wild crop relatives deserve serious attention for domestication, especially for the utilization of their phytomedicines and nutraceuticals, bioenergy production, soil reclamation, and the phytoremediation of ecology and environment. While these vastly positive impacts of wild crop relatives on the development and deployment of new varieties for various purposes in the major crop plants of the world agriculture, along with a few negative potential concerns, are envisaged the need for reference books with comprehensive deliberations on the wild relatives of all the major field and plantation crops and fruit and forest trees is indeed imperative. This was the driving force behind the inception and publication of this series.

Unlike the previous six book projects I have edited alone or with co-editors, this time it was very difficult to formulate uniform outlines for the chapters of this book series for several obvious reasons. Firstly, the status of the crop relatives is highly diverse. Some of them are completely wild, some are sporadically cultivated and some are at the initial stage of domestication for specific breeding objectives recently deemed essential. Secondly, the status of their conservation varies widely: some have been conserved, characterized and utilized; some have been eroded completely except for their presence in their center(s) of origin; some are at-risk or endangered due to genetic erosion, and some of them have yet to be explored. The third constraint is the variation in their relative worth, e.g. as academic model, breeding resource, and/or potential as "new crops."

The most perplexing problem for me was to assign the chapters each on a particular genus to different volumes dedicated to crop relatives of diverse crops grouped based on their utility. This can be exemplified with *Arabidopsis*, which has primarily benefited the Brassicaceae crops but also facilitated genetic analyses and improvement in crop plants in other distant families; or with many wild relatives of forage crops that paved the way for the genetic analyses and breeding of some major cereal and millet crops. The same is true for wild crop relatives such as *Medicago truncatula*, which has paved the way for in-depth research on two crop groups of diverse use: oilseed and pulse crops belonging to the Fabaceae family. The list is too long to enumerate. I had no other choice but to compromise and assign the genera of crop relatives in a volume on the crop group to which they are taxonomically the closest and to which they have relatively greater contributions. For example, I placed the chapter on genus *Arabidopsis* in the volume on oilseeds, which deals with the wild relatives of Brassicaceae crops amongst others.

However, we have tried to include deliberations pertinent to the individual genera of the wild crop relatives to which the chapters are devoted. Descriptions of the geographical locations of origin and genetic diversity, geographical distribution, karyotype and genome size, morphology, etc. have been included for most of them. Their current utility status – whether recognized as model species, weeds, invasive species or potentially cultivable taxa – is also delineated. The academic, agricultural, medicinal, ecological, environmental and industrial potential of both the cultivated and/or wild allied taxa are discussed.

The conservation of wild crop relatives is a much discussed yet equally neglected issue albeit the in situ and ex situ conservations of some luckier species were initiated earlier or are being initiated now. We have included discussions on what has happened and what is happening with regard to the conservation of the crop relatives, thanks to the national and international endeavors, in most of the chapters and also included what should happen for the wild relatives of the so-called new, minor, orphan or future crops.

The botanical origin, evolutionary pathway and phylogenetic relationship of crop plants have always attracted the attention of plant scientists. For these studies morphological attributes, cytological features and biochemical parameters were used individually or in combinations at different periods based on the availability of the required tools and techniques. Access to different molecular markers based on nuclear and especially cytoplasmic DNAs that emerged after 1980 refined the strategies required for precise and unequivocal conclusions regarding these aspects. Illustrations of these classical and recent tools have been included in the chapters.

Positioning genes and defining gene functions required in many cases different cytogenetic stocks, including substitution lines, addition lines, haploids, monoploids and aneuploids, particularly in polyploid crops. These aspects have been dealt in the relevant chapters. Employment of colchiploidy, fluorescent or genomic in situ hybridization and Southern hybridization have reinforced the theoretical and applied studies on these stocks. Chapters on relevant genera/species include details on these cytogenetic stocks.

Wild crop relatives, particularly wild allied species and subspecies, have been used since the birth of genetics in the twentieth century in several instances such as studies of inheritance, linkage, function, transmission and evolution of genes. They have been frequently used in genetic studies since the advent of molecular markers. Their involvement in molecular mapping has facilitated the development of mapping

populations with optimum polymorphism to construct saturated maps and also illuminating the organization, reorganization and functional aspects of genes and genomes. Many phenomena such as genomic duplication, genome reorganization, self-incompatibility, segregation distortion, transgressive segregation and defining genes and their phenotypes have in many cases been made possible due to the utilization of wild species or subspecies. Most of the chapters contain detailed elucidations on these aspects.

The richness of crop relatives with biotic and abiotic stress resistance genes was well recognized and documented with the transfer of several alien genes into their cultivated counterparts through wide or distant hybridization with or without employing embryo-rescue and mutagenesis. However, the amazing revelation that the wild relatives are also a source of yield-related genes is a development of the molecular era. Apomictic genes are another asset of many crop relatives that deserve mention. All of these past and the present factors have led to the realization that the so-called inferior species are highly superior in conserving desirable genes and can serve as a goldmine for breeding elite plant varieties. This is particularly true at a point when natural genetic variability has been depleted or exhausted in most of the major crop species, particularly due to growing and promoting only a handful of so-called high-yielding varieties while disregarding the traditional cultivars and landraces. In the era of molecular breeding, we can map desirable genes and polygenes, identify their donors and utilize tightly linked markers for gene introgression, mitigating the constraint of linkage drag, and even pyramid genes from multiple sources, cultivated or wild taxa. The evaluation of primary, secondary and tertiary gene pools and utilization of their novel genes is one of the leading strategies in present-day plant breeding. It is obvious that many wide hybridizations will never be easy and involve near-impossible constraints such as complete or partial sterility. In such cases gene cloning and gene discovery, complemented by intransgenic breeding, will hopefully pave the way for success. The utilization of wild relatives through traditional and molecular breeding has been thoroughly enumerated over the chapters throughout this series.

Enormous genomic resources have been developed in the model crop relatives, for example *Arabidopsis thaliana* and *Medicago truncatula*. BAC, cDNA and EST libraries have also been developed in some other crop relatives. Transcriptomes and metabolomes have also been dissected in some of them. However, similar genomic resources are yet to be constructed in many crop relatives. Hence this section has been included only in chapters on the relevant genera.

In this book series, we have included a section on recommendations for future steps to create awareness about the wealth of wild crop relatives in society at large and also for concerns for their alarmingly rapid decrease due to genetic erosion. The authors of the chapters have also emphasized on the imperative requirement of their conservation, envisaging the importance of biodiversity. The importance of intellectual property rights and also farmers' rights as owners of local landraces, botanical varieties, wild species and subspecies has also been dealt in many of the chapters.

I feel satisfied that the authors of the chapters in this series have deliberated on all the crucial aspects relevant to a particular genus in their chapters.

I am also very pleased to present many chapters in this series authored by a large number of globally reputed leading scientists, many of whom have contributed to the development of novel concepts, strategies and tools of genetics, genomics and breeding and/or pioneered the elucidation and improvement of particular plant

genomes using both traditional and molecular tools. Many of them have already retired or will be retiring soon, leaving behind their legacies and philosophies for us to follow and practice. I am saddened that a few of them have passed away during preparation of the manuscripts for this series. At the same time, I feel blessed that all of these stalwarts shared equally with me the wealth of crop relatives and contributed to their recognition and promotion through this endeavor.

I would also like to be candid with regard to my own limitations. Initially I planned for about 150 chapters devoted to the essential genera of wild crop relatives. However, I had to exclude some of them either due to insignificant progress made on them during the preparation of this series, my failure to identify interested authors willing to produce acceptable manuscripts in time or authors' backing out in the last minute, leaving no time to find replacements. I console myself for this lapse with the rationale that it is simply too large a series to achieve complete satisfaction on the contents. Still I was able to arrange about 125 chapters in the ten volumes, contributed by nearly 400 authors from over 40 countries of the world. I extend my heartfelt thanks to all these scientists, who have cooperated with me since the inception of this series not only with their contributions, but also in some cases by suggesting suitable authors for chapters on other genera. As happens with a mega-series, a few authors had delays for personal or professional reasons, and in a few cases, for no reason at all. This caused delays in the publication of some of the volumes and forced the remaining authors to update their manuscripts and wait too long to see their manuscripts in published form. I do shoulder all the responsibilities for this myself and tender my sincere apologies.

Another unique feature of this series is that the authors of chapters dedicated to some genera have dedicated their chapters to scientists who pioneered the exploration, description and utilization of the wild species of those genera. We have duly honored their sincere decision with equal respect for the scientists they rightly reminded us to commemorate.

Editing this series was, to be honest, very taxing and painstaking, as my own expertise is limited to a few cereal, oilseed, pulse, vegetable, and fruit crops, and some medicinal and aromatic plants. I spent innumerable nights studying to attain the minimum eligibility to edit the manuscripts authored by experts with even life-time contributions on the concerned genera or species. However, this indirectly awakened the "student-for-life" within me and enriched my arsenal with so many new concepts, strategies, tools, techniques and even new terminologies! Above all, this helped me to realize that individually we know almost nothing about the plants on this planet! And this realization strikingly reminded me of the affectionate and sincere advice of Dr. Norman Borlaug to keep abreast with what is happening in the crop sciences, which he used to do himself even when he had been advised to strictly limit himself to bed rest. He was always enthusiastic about this series and inspired me to take up this huge task. This is one of the personal and professional reasons I dedicated this book series to him with a hope that the present and future generations of plant scientists will share the similar feelings of love and respect for all plants around us for the sake of meeting our never-ending needs for food, shelter, clothing, medicines, and all other items used for our basic requirements and comfort. I am also grateful to his granddaughter, Julie Borlaug, for kindly extending her permission to dedicate this series to him.

I started editing books with the 7-volume series on Genome Mapping and Molecular Breeding in Plants with Springer way back in 2005, and I have since

edited many other book series with Springer. I always feel proud and satisfied to be a member of the Springer family, particularly because of my warm and enriching working relationship with Dr. Sabine Schwarz and Dr. Jutta Lindenborn, with whom I have been working all along. My special thanks go out to them for publishing this "dream series" in an elegant form and also for appreciating my difficulties and accommodating many of my last-minute changes and updates.

I would be remiss in my duties if I failed to mention the contributions of Phullara – my wife, friend, philosopher and guide – who has always shared with me a love of the collection, conservation, evaluation, and utilization of wild crop relatives and has enormously supported me in the translation of these priorities in my own research endeavors – for her assistance in formulating the contents of this series, for monitoring its progress and above all for taking care of all the domestic and personal responsibilities I am supposed to shoulder. I feel myself alien to the digital world that is the sine qua non today for maintaining constant communication and ensuring the preparation of manuscripts in a desirable format. Our son Sourav and daughter Devleena made my life easier by balancing out my limitations and also by willingly sacrificing the spare amount of time I ought to spend with them. Editing of this series would not be possible without their unwavering support.

I take the responsibility for any lapses in content, format and approach of the series and individual volumes and also for any other errors, either scientific or linguistic, and will look forward to receiving readers' corrections or suggestions for improvement.

As I mentioned earlier this series consists of ten volumes. These volumes are dedicated to wild relatives of Cereals, Millets and Grasses, Oilseeds, Legume Crops and Forages, Vegetables, Temperate Fruits, Tropical and Subtropical Fruits, Industrial Crops, Plantation and Ornamental Crops, and Forest Trees.

This volume "Wild Crop Relatives – Genomic and Breeding Resources: Legume Crops and Forages" includes 15 chapters dedicated to *Arachis*, *Cajanus*, *Chenopodium*, *Cicer*, *Glycine*, *Lathyrus*, *Lens*, *Lotus*, *Lupinus*, *Medicago*, *Phaseolus*, *Pisum*, *Trifolium*, *Vicia*, and *Vigna*. The chapters of this volume were authored by 47 scientists from 9 countries of the world, namely Australia, Bolivia, Brazil, India, Japan, New Zealand, Poland, UK, and the USA.

It is my sincere hope that this volume and the series as a whole will serve the requirements of students, scientists and industries involved in studies, teaching, research and the extension of legume crops and forages with an intention of serving science and society.

Clemson, USA Chittaranjan Kole

Contents

Abbreviations

α-EST	α-esterase
ψleaf	Leaf water potential
1C	DNA content of the haploid genome
2C	DNA content of the diploid genome
AB	Advanced backcross
ACP	Acid phosphatase
AFLP	Amplified fragment length polymorphism
ALC	Australian Lupin Collection
AM	Association mapping
AND	Andean subregion of South America
ANF	Anti-nutritional factor
APS	Adenosine 5′ phosphosulfate
ARS	Agricultural Research Service
ASL	Apical segment length
ASPADERUC	Association for Rural Development in Cajamarca
AS-PCR	Allele-specific PCR
ATL	Atlantic subregion of South America
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BBSRC	Biotechnology and Biological Sciences Research Council
BC	Backcross
BC_1F_1	First filial generation of backcross with the first parent
BES	BAC-end sequence
BGM	Botrytis gold mold
BGMV	Bean golden mosaic virus
BP	Branching pattern
BSL	Basal segment length
BYMV	Bean yellow mosaic virus
CCD	Charge coupled device
cDNA	Complementary DNA
CENARGEN	Centro Nacional de Pesquisas de Recursos Genéticos e Biotecnologia (Brazilian National Center for Genetic Resources and Biotechnology)
CEW	Corn earworm
CGIAR	Consultative Group on International Agricultural Research
CI	Centromere index

CIAT	Centro Internacional de Agricultura Tropical (International Center for Tropical Agriculture)
CIP	International Potato Center
cM	Centi-Morgan
CMS	Cytoplasmic male sterility
CMV	Cucumber mosaic virus
CONDESAN	Consortium for the Sustainable Development of the Andean Ecoregion
cpDNA	Chloroplast-DNA
CSIRO	Commonwealth Scientific and Industrial Research Organization
cv.	Cultivar
CWR	Crop wild relatives
CyTed	Ibero-American Development Programme for Science and Technology
D	Nei's genetic distance
DAAL	Disomic alien addition line
DAFWA	Department of Agriculture and Food Western Australia
DAP	Days after pollination
DAPI	4′-6-Diamidino-2-phenylindole
DArT	Diversity arrays technology
DAS	Days after sowing
DE	Days to emergence
DF	Days to 50% flowering
DIA	Diafarase
DM	Days to maturity
dr	Diffusion resistance
eIF	Eukaryotic initiation factor
ELS	Early leaf spot
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária (Brazilian Agricultural Research Corporation
EMS	Ethylmethane sulphonate
EST	Expressed sequence tag
ETS	External transcribed spacer region
F_1	First filial generation
F_2	Second filial generation
FAO	Food and Agriculture Organization
FISH	Fluorescence in situ hybridization
FLM	Flowers on main stem
FPC	Fingerprint contig
FTP	File transfer protocol
FYM	Farm yard manure
GA/ GA_3	Gibberellic acid
GH	Growth habit
GISH	Genomic in situ hybridization
GM	Genetic modification/genetically modified
GOT	Glutamic-oxaloacetic transaminase
GP	Gene pool
GRIN	Germplasm Resource Information Network
HSW	100-seed weight
HYH	Hypanthium hairiness

IAA	Indole acetic acid
IBONE	Instituto de Botánica del Nordeste (Botanical Institute of the Northeast)
IBPGR	International Board for Plant Genetic Resources (now IPGRI)
ICA	Instituto Colombiano Agropecuario (Colombian Agricultural Institute)
ICARDA	International Center for Agricultural Research in the Dry Areas
ICRISAT	International Crops Research Institute for the Semi Arid Tropics
ILDIS	International Legume Database and Information Service
IMGAG	International Medicago Genome Annotation Group
INIA	Instituto Nacional de Innovación Agraria
INIA-CGNA	Instituto Nacional de Investigación Agropecuarias – Centro de Genómica Nutricional Agro Acuícola
INIAP	Instituto Nacional de Investigación Agropecuarias
INTA	National Institute for Agricultural Technology
IPGRI	International Plant Genetic Resources Institute (Now Biodiversity International)
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
IRLC	Inverted repeat-lacking clade
ISRIC	World Soil Information
ISSR	Inter-simple sequence repeat
ITIS	International Taxonomic Identification System
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
K	Number of proposed ancestral populations
KEGG	Kyoto Encyclopedia of Genes and Genomes
KN	Kinetin
KOG	EuKaryotic Orthologous Groups database
LB	Leaflet bristle
LBMSPL	Leaflet bristle on margin of main stem and primary laterals
LBPL	Leaflet bristle on primary lateral
LC	Leaflet color
LFI	Length of first isthmus
LH	Leaflet hairiness
LHMSPL	Leaflet hairiness on margin of main stem and primary laterals
LHPL	Leaflet hairiness on primary lateral
LINE	Long interspersed (repeated DNA) element
Lj	*Lotus japonicus*
*Lj*R	*Lotus japonicus* repeat region
LLAPL	Leaflet length of the apical primary lateral
LLBMS	Leaflet length on basal main stem
LLBPL	Leaflet length on basal primary lateral
LLS	Late leaf spot
LMHLMS	Leaflet midrib hairiness on lower main stem
LMHLPL	Leaflet midrib hairiness on lower primary lateral
LMHUMS	Leaflet midrib hairiness on upper main stem
LMHUPL	Leaflet midrib hairiness on upper primary lateral
LOTASSA	Lotus adaptation and sustainability in South America
LRR	Leucine-rich repeat
LSAMS	Leaflet shape on apical main stem

LSAPL	Leaflet shape on apical primary lateral
LSBMS	Leaflet shape on basal main stem
LSBPL	Leaflet shape on basal primary lateral
LSMS	Leaflet surface on main stem
LSPL	Leaflet surface on primary lateral
LTR	Long terminal repeat
LTSMS	Leaflet tip shape of the main stem
LTSPL	Leaflet tip shape of primary lateral
LWAMS	Leaflet width of apical main stem
LWAPL	Leaflet width of apical primary lateral
LWBMS	Leaflet width on basal main stem
LWBPL	Leaflet width on basal primary lateral
MAAL	Monosomic alien addition line
MAC	Mountain range of North and Central America
MAS	Marker-assisted selection
MFFGC	Margot Forde Forage Germplasm Centre
MFLP	Microsatellite-anchored fragment length polymorphism
MIPS	Munich Information Center for Protein Sequences
miRNA	Micro-RNA
MLG	Molecular linkage group
MS	Murashige and Skoog (medium)
MS	Mass-spectrometry
MSH	Main stem hairiness
MSHT	Main stem hair type
MSHt	Main stem height
MST	Main stem thickness
MST	Mass-spectral metabolite tag
Mt	*Medicago truncatula*
*Mt*R	*Medicago truncatula* repeat region
Mya	Million years ago
NAR	No adventitious roots
NARS	National Agricultural Research System
NBPGR	National Bureau of Plant Genetic Resources
NBRI	National Botanical Research Institute
NBS	Nucleotide-binding site
NCBI	National Center for Biotechnology Information
NIAS	National Institute of Agrobiological Sciences
NLB	Number of lateral branches
NLL	Narrow leafed lupin
NMU	N-Nitroso-N-methyl urea
NOR	Nucleolus organizer region
NPGS	National Plant Germplasm System
NPMSPL	Nature of petiole on main stem and on primary laterals
NSBP	Number of segments between pods
NSP	Non-starch polysaccharides
NSTPL	Nature of stipule on primary lateral
NTS	Non-transcribed spacer
ODAP	β-N-Oxalyl-l-α,β-diaminopropanoic acid
PAGE	Polyacrylamide gel electrophoresis

PB	Pod beak
PBMS	Petiole bristle on main stem
PBPL	Petiole bristle on primary lateral
PC	Principal component
PCGIN	Pulse Crop Genetic Improvement Network
PCR	Polymerase chain reaction
PDL	Pod length
PDR	Pea dispersed repeat
PDW	Pod width
PEBV	Pea early browning virus
PG	Peg growth
PGMS	Petiole groove on main stem
PGP	Peg pigmentation
PGPL	Petiole groove on primary lateral
PHMS	Petiole hairiness on main stem
PHPL	Petiole hairiness on primary lateral
PL	Peg length
PLMS	Petiole length on main stem
PLPL	Petiole length of primary lateral
PMS	Pigmentation on main stem
PMV	Peanut mottle virus
PPi	Pyrophosphate
PR	Pod reticulation
PRINS	Primed in situ labeling
PROINPA	Foundation for the Promotion and Investigation of Andean Products
PStV	Peanut stripe virus
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RG	Root growth
RGA	Resistance gene analog
RGM	Rachis groove on main stem
RGPL	Rachis groove on primary lateral
RH	Relative humidity
RI	Relative injury
RIL	Recombinant inbred line
RLMS	Rachis length of the main stem
RLPL	Rachis length of primary lateral
RNAi	RNA interference
rRNA	Ribosomal RNA
RuBisCo	Ribulose biphosphate carboxylase/oxygenase
SAGE	Serial analysis of gene expression
SALMS	Stipule adnation length on main stem
SALPLB	Stipule adnation length on primary lateral branch
SAT	Satellite chromosome
SAWMS	Stipule adnation width on main stem
SAWPLB	Stipule adnation width on primary lateral branch

SBMMS	Stipule bristles on margin of the main Stem
SBMPL	Stipule bristle on the margin of primary lateral
SBOMS	Stipule bristles outside of the main stem
SBOPLB	Stipule bristle outside primary lateral branch
SC	Seed color
SCAR	Sequence-characterized amplified region
SCMR	SPAD Chlorophyll meter reading
SDL	Seed length
SDS	Sodium dodecyl sulfate
SDW	Seed width
SEM	Scanning electron microscopy
SEN	Southeastern subregion of North and Central America
SHMMS	Stipule hairiness on margin of the main stem
SHMPLB	Stipule hairiness on margin of primary lateral branch
SHOMS	Stipule hairiness on outside of the main stem
SHOPL	Stipule hairiness on outside primary lateral
SLA	Specific leaf area
SLMS	Stipule length on main stem
SLPLB	Stipule length on primary lateral branch
SM	Stem modification
SMD	Sterility mosaic disease
SMP®	Solid Matrix Priming®
SNP	Single nucleotide polymorphism
SPAD	Soil plant analysis development
SPC	Standard petal color
SPL	Standard petal length
SPMBF	Standard petal markings on back face
SPMFF	Standard petal markings on front face
SPW	Standard petal width
SRAP	Sequence related amplified polymorphism
SSAP	Sequence-specific amplified polymorphism
SSR	Simple sequence repeat
STMS	Sequence tagged microsatellite site
STS	Sequence tagged site
TAC	Transformation competent artificial chromosome
TC	Tentative consensus
TIILING	Targeting induced local lesions in genomes
tr	Transpiration rate
tRNA	Transfer RNA
TSWV	Tomato spotted wilt virus
ULCL	Upper lip calyx lobation
UN	United Nations
UNALM	National Agrarian University of La Molina (Peru)
UNAP	National University of the Altiplano – Puno (Peru)
USDA	US Department of Agriculture
UV	Ultra violet light
VIGS	Virus-induced gene silencing
WPBS	Welsh Plant Breeding Station
WPC	Wing petal color

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Chapter 1
Arachis

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1.1 Introduction

Crop wild relatives (CWR) are wild plant taxa that have an indirect use derived from their relatively close genetic relationships to crops (Maxted et al. 2006). An understanding of the taxonomic and evolutionary relationships between cultigens and their wild relatives is prerequisite for the exploitation of wild relatives in crop improvement programs (Hawkes 1977). In the past, several reviews on the use of wild relatives for crop improvement have been published, which demonstrated greatest benefit towards improving the levels of resistance to pests and diseases in several crops including groundnut (Harlan 1976; Stalker 1980; Goodman et al. 1987; Lenne and Wood 1991; Hoisington et al. 1999; Dwivedi et al. 2003). Hajjar and Hodgkin (2007) documented information on the presence of genes from CWR in released cultivars of CGIAR mandate crops, demonstrating that there has been steady increase in the rate of release of cultivars containing genes from CWR. More recently, it has also been demonstrated that CWR have contributed alleles associated with increased fruit/grain yield and improved seed quality, predominantly in tomato and rice, and resistance to drought and salinity in wheat [reviewed in Dwivedi et al. (2008)].

Groundnut (*Arachis hypogaea* L.) originated in South America and is widely grown (113 countries) throughout tropical, subtropical and warm temperate regions (40°N to 40°S). Worldwide, groundnut is next in importance after soybean and rapeseed, with an annual production of 38.2 million tons and average productivity of 1.5 ton ha^{-1} (FAO 2008). The seeds are rich in oil and protein and are eaten in a variety of forms. About two-thirds of global production is crushed for extracting vegetable oil. The remaining one-third is used in the form of edible product and as seed. The cake obtained after oil extraction is used as protein-rich meal for livestock or for making other food products. The haulms are an important source of good quality animal fodder. Some of the perennial wild species, such as *A. glabrata* from the section Rhizomatosae, have been used to develop several commercial tropical forage cultivars, including the Florigraze and Arbrook in the USA that are used as an alternative to alfalfa because of their high levels of proteins and resistance to pest and diseases (Prine et al. 1981, 1986; French et al. 1994). Likewise, in Australia, *A. glabrata* is valued as high-quality forage having the ability to spread through swards of aggressive summer-growing grass species (Bowman et al. 1998). In addition, groundnut helps to improve soil fertility through biological nitrogen fixation.

Rust, early leaf spot, and late leaf spot are the most common and widely distributed foliar diseases of groundnut worldwide, while leaf minor is common in South Asia; army worm (*Spodoptera*) and bacterial wilt in South-east Asia; groundnut rosette disease and termite in Africa; and nematode, corn earworm, lesser corn stock borer, and southern corn rootworm in North America. Some insects are also the vectors of important viral diseases – *Thrips palmi* for peanut bud necrosis virus, *Frankliniella occidentalis* and *F. fusca* for tomato-spotted wilt virus and *Aphis crassivora* for groundnut rosette virus. In addition to biotic stresses, the crop is also adversely affected by drought, salinity, low availability of phosphorus under acidic soils and nonavailability of iron in calcareous soils in many

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parts of the world. Aflatoxin contamination is the major problem adversely affecting the groundnut seed quality. All these factors either alone or in combination adversely affect the yield and/or quality worldwide, necessitating the identification and utilization of resistance sources to enhance and sustain groundnut production. With regard to several pests and diseases, the level of resistance required is either not present or available only at very low levels in cultivated groundnut, while very high levels of resistance to pests and diseases have been reported in many wild *Arachis* relatives [reviewed in Dwivedi et al. (2003)].

The cultivated groundnut, *A. hypogaea*, belongs to the section *Arachis*, which also contains its tetraploid progenitor *A. monticola* Krapov. and Rigoni (Favero et al. 2006), and 29 wild diploid species that are cross-compatible with *A. hypogaea*. *A. ipaënsis* and *A. duranensis* have been suggested as putative B- and A-genome donors, respectively, of the cultivated peanut (Kochert et al. 1996; Seijo et al. 2004; Favero et al. 2006). More recently, Seijo et al. (2007) used the double genomic in situ hybridization (GISH) technique on seven diploid species that harbored either the A- or B-genome, to provide further evidence that *A. duranensis* (A-genome) and *A. ipaënsis* (B-genome) are the best candidates for the genome donors of cultivated groundnut as both yielded the most intense and uniform hybridization pattern when tested against the corresponding chromosome subsets of *A. hypogaea*. Further, all the presently known subspecies and varieties of *A. hypogaea* have arisen from a unique allotetraploid plant population, or alternatively, from different tetraploid populations that originated from the same two diploid species.

Singh and Simpson (1994) have classified the genetic variability in the genus *Arachis* into four genepools: primary gene pool (landraces of *A. hypogaea* and its wild form *A. monticola*), secondary gene pool (diploid species from section *Arachis* that are cross-compatible with *A. hypogaea*), tertiary gene pool (species of section *Procumbentes* that are weekly cross-compatible with *A. hypogaea*) and the fourth gene pool (wild *Arachis* species classified into seven other sections). While interspecific crosses involving some species from secondary gene pool have been successful in groundnut, it is more difficult to cross species from tertiary and forth gene pool, for which, techniques such as in vitro culture of ovule and embryo is a must to produce viable hybrids (see Sect. 1.7).

This review is devoted to the use of wild *Arachis* for the improvement of *A. hypogaea* (cultivated groundnut) with the focus on conservation and regeneration of wild *Arachis* genetic resources; geographical distribution and the need to expedite collection of those species not present in genebanks before these are lost due to climate change or habitat disturbances in South America; differences in ploidy levels, genomes, and crossing relationships; descriptors used to characterize *Arachis* species; sources of resistance to biotic and abiotic stresses and for seed quality; barriers to interspecific hybridization; genomic resources developed to facilitate introgression of beneficial traits from wild *Arachis* to *A. hypogaea*; approaches to interspecific gene transfer and use of genetic markers to demonstrate the introgression of traits from wild *Arachis* species; the elite germplasm and cultivars developed using wild *Arachis* species; and new approaches to unlock the genetic variation from wild relatives using appropriate genetic and genomic resources.

1.2 Wild *Arachis* Species

1.2.1 Geographical Distribution

Arachis is exclusively a genus of South America and consists of nine sections that comprise 80 annual and perennial species (Krapovickas and Gregory 1994; Valls and Simpson 2005). It belongs to the family Leguminosae-Papilionoideae, tribe Aeschynomeneae and subtribe Stylosanthinae, and is restricted to Argentina, Bolivia, Paraguay and Uruguay. The *Arachis* section species occur in Brazil (mostly in the west central region) followed by Paraguay, Argentina and Uruguay. Wild *Arachis* species occur both in open and shaded areas, ranging from near to the equator to 34°S and from sea level to an attitude of almost 1,600 m. Because of the geocarpic nature of the fruit, species distribution generally follows major river valleys. Infrageneric groups may be closely associated with specific drainage basins, such as members of the section Triseminatae are found in the São Francisco,

while species of the section *Arachis* in the drainage basin of the river Paraguay and also in the Amazon drainage basin. Some overlap in distribution does occur for the sections Arachis, Erectoides, Rhizomatosae and Extranervosae (Gregory et al. 1973; Valls 1983; Valls et al. 1985). Species in the section Arachis are distributed in Argentina, Bolivia, Brazil, Uruguay and Paraguay, from the southern extreme of the genus along the river Uruguay to the eastern most extreme of the genus in Bolivia and Argentina and northeastwards across the Brazilian Highlands. Section Heteranthae contains six species and is endemic to the north-eastern highlands of Brazil, while section *Trierectoides* contains two species, *A. guaranitica* and *A. tuberosa*, and is geographically restricted to a narrow distribution range in Brazil (one population of *A. guaranitica* is also reported from Paraguay). Species in section *Caulorhizae* including *A. pintoi* and *A. repens* are endemic to Brazil and centered in the eastern Brazilian highlands with scattered populations found towards the highlands of Mato Grosso do Sul. Section *Procumbentes* species are distributed where the borders of Paraguay, Bolivia and Brazil come together, near an area known as Pantanal while Erectoides section species are restricted largely in the Brazilian Province of Mato Grosso do Sul stretching southwards in Paraguay. Section *Extranervosae* species are also endemic to Brazil, inhabiting the Brazilian Highlands north and west of Mato Grosso do Sul, spreading across the Brazilian Plateau as far as 5°S. Section *Triseminatae* is endemic to the north-eastern Brazilian Highlands, while section *Rhizomatosae* species inhabit areas surrounding the Parana basin, and southwards through Paraguay, Argentina and into Uruguay, following the Rio Paraguay and meeting the Rio Uruguay (Ferguson et al. 2005).

1.2.2 *Ex Situ Conservation of Wild* Arachis *Genetic Resources and Priority Areas for Future Collection*

The major centers of conservation of wild *Arachis* species are in India, Brazil, USA, Argentina and Columbia, together holding ~2,800 accessions (Table 1.1). ICRISAT developed *Arachis* house, an open space fixed with a large cylindrical concrete structure (75 cm high, 90 cm in inner ring diameter, and of 5 cm ring thickness) with a ring-to-ring distance of 52.5 cm, for regenerating the seeds of wild *Arachis* species (Fig. 1.1). These rings are filled with about 0.5 m^3 pasteurized [3 cycles of 1 h each at 82°C and 34.5×10^3 Pa (5 psi)] soil mixture (soil, sand and FYM in 3:2:1 ratio). Five to six plants can be accommodated in one ring. After harvesting the pods at maturity, the remnant pods/seeds are visually collected and destroyed to avoid contamination with the next seed lot. The rings are kept fallow for 2–3 months and 2–3 irrigations are provided to allow remnant seeds, if any, to germinate, which are destroyed before the next seed lot is sown.

Preservation of wild *Arachis* species, in general, is difficult, particularly for accessions that produce a few seeds, and especially the section *Rhizomatosae* species, which are maintained as vegetative materials in greenhouse (Stalker and Simpson 1995). An international cooperative effort is underway to ensure that these vegetatively propagated species are maintained in multiple environments for conservation to minimize their loss (Singh and Simpson 1994). This effort involves the cooperation of USDA, North Carolina State University, Texas A&M University, ICRISAT, the Brazilian Corporation for Agricultural Research

Table 1.1 Major holdings of wild *Arachis* species accessions in genebank

Country	Institute	# Accessions
Argentina	Instituto Botánico del Nordeste, Universidad Nacional de Nordeste (IBONE)	109
Australia	Australian Tropical Crops and Forages Genetic Resources Centre	65
Brazil	Embrapa Recursos Geneticos e Biotecnologia (CENARGEN) Instituto AgronOmico de Campinas	450
Colombia	Centro Internacional de Agricultura Tropical (CIAT)	243
	Centro de Investigaciones de Nataima, Instituto Colombiano Agropecuario (ICA)	225
India	International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad	453
USA	USDA, Griffin, USA	498
	Texas A&M University, USA	798



Fig. 1.1 (**a**) *Arachis* species grown in large cylindrical concrete structures in the *Arachis* House, ICRISAT, Patancheru, India. (**b**) Scientists examining wild *Arachis* species growing in these structures

(EMBRAPA), the Brazilian National Center for Genetic Resources and Biotechnology (CENARGEN), the Argentina National Institute of Agricultural Technology (INTA) and the Argentina Botanical Institute of the Northeast (IBONE).

Spatial analysis of in situ wild relatives distribution using species richness (areas potentially high in species richness), proximity to existing accessions (areas most distant from the existing collections, thus targeting geographical gaps in existing collections), proximity to protected areas (areas most distant from protected areas), and risk to genetic erosion (areas with the greatest risk of genetic erosion) revealed that hotspot regions for the wild *Arachis* species' richness in Brazil include Serra Geral de Goias, north-east of Brasilia, the region west of Campo Grande in Mato Grosso do Sul, and the region 170 km south of Cuiaba, in Mato Grosso. In addition, 300 km south-east of the city of Cuiabá, near Pedro Gomes, has also been identified as a species-rich area where species such as *A. cryptopotamica*, *A. diogoi*, *A. glabrata*, *A. helodes*, *A. hoehnei*, *A. kuhlmannii*, *A. lutescens*, *A. matiensis*, *A. stenosperma* and *A. subcoriacea* exist sympatrically (Jarvis et al. 2002, 2003). Another area is the municipality of Parauna in the state of Goias (Brazil), where only *A. prostata* and *A. glabrata* were collected in the past, as it is predicted that as many as six different species may be found in this region, although the land in this region is predominantly agricultural (Jarvis et al. 2003). Likewise, considering anthropogenic influences as a risk to genetic erosion, some areas in Bolivia, where about five species potentially lie sympatrically (Jarvis et al. 2002), have also been highlighted for future collections. Further, Jarvis et al. (2003) emphasized the need for more effort to collect and conserve species belonging to B-genome such as *A. williamsii*, *A. cruziana* and *A. ipaënsis* along with *A. martii*, *A. Pietrarellii*, *A. vallsii* and *A. monticola*, which are also under the risk of extinction.

1.2.3 *Climate Change and Habitat Disturbances a Threat to Wild* Arachis *in South America*

Climate change poses serious impacts on biodiversity and has a potential to wipe out biodiversity. Wild relatives of groundnut are at risk of extinction, threatening a valuable repository of genes needed for the improvement of the cultivated groundnut. In a recent study, it is predicted that in the next 50 years, as many as 61% of the 51 wild groundnut species studied would become extinct, as a result of climate change (Jarvis et al. 2008). The areas, where wild *Arachis* species are most at risk, include Santa Cruz to Cuiba and along the Andean fringe in the south of Santa Cruz (Bolivia), eastern Bolivia, Paraguay and south-western Brazil. In recent years, there have been intensive developmental activities in these regions, thus disturbing the remote and fragile environments (Jarvis et al. 2002). Most of the wild species generally occur in the region under intensive environmental disturbance, which has led to habitat destruction and genetic erosion. Some *Arachis* species are particularly threatened by habitat loss. The species, which are most restricted in distribution, include *A. archeri*, *A. setinervosa*, *A. marginata*, *A. hatschbachii*, *A. appressipila*, *A. villosa*, *A. cryptopotamica*, *A. helodes*, *A. magna* and *A. gracilis*. Their distribution is limited to less than 10,000 km^2 of climatically suitable wild habitat, while *A. burkatii*, *A. triseminata*, *A. tuberosa* and *A. Dardani* remain above 10,000 km^2, but their distribution has been reduced by more than 75% because of agricultural land use (Jarvis et al. 2003).

1.2.4 *Ploidy Levels and Genome Variations Among Wild* Arachis *Species*

The cultivated groundnut is a tetraploid with chromosome number, $2n = 40$, and genome size 2,813 Mbp. The first chromosome count reported for a wild species was $2n = 40$ for *A. glabrata* (Gregory 1946). Mendes (1947) published the chromosome count of $2n = 20$ for *A. oteroi*, *A. benthami*, *A. archeri*, *A. major* and *A. villosulicarpa*, which gave the first indication of the existence of $2n = 20$ and $2n = 40$ chromosomes in the genus *Arachis*. Later on, several studies confirmed the existence of $2n = 2x = 20$ and $2n = 4x = 40$, with basic chromosome number $n = 10$ (Krapovickas and Rigoni 1957; Krapovickas and Gregory 1960; Conagin 1964; Smartt 1965; also see Table 1.2). Polyploidy has apparently arisen independently at least twice in the genus, in the sections *Arachis* and *Rhizomatosae*. In the section *Arachis*,

Table 1.2 Chromosome counts in species belonging to the genus *Arachis* (Krapovickas and Gregory 1994; Valls and Simpson 2005)

Species	Chromosome number (2*n*)	Species	Chromosome number (2*n*)
Section *Trierectoides*			
A. guaranitica	20	*A. tuberosa*	20
Section *Erectoides*			
A. martii	20	*A. gracilis*	20
A. brevipetiolata	20	*A. hermannii*	20
A. oteroi	20	*A. archeri*	20
A. hatschbachii	20	*A. stenophylla*	20
A. cryptopotamica	20	*A. paraguariensis* spp. *capibarensis*	20
A. major	20	*A. paraguariensis* spp. *paraguariensis*	20
A. benthamii	20	*A. porphyrocalyx*	20
A. douradiana	20		
Section *Extranervosae*			
A. setinervosa	20	*A. retusa*	20
A. macedoi	20	*A. burchellii*	20
A. marginata	20	*A. pietrarellii*	20
A. prostrata	20	*A.villosulicarpa*	20
A. lutescens	20	*A. submarginata*	20
Section *Triseminatae*			
A. triseminata	20		
Section *Heteranthae*			
A. giacomettii	20	*A. dardani*	20
A. sylvestris	20	*A. interrupta*	20
A. pusilla	20	*A. seridoënsis*	20
Section *Caulorrhizae*			
A. repens	20	*A. pintoi*	20
Section *Procumbentes*			
A. lignosa	20	*A. appressipila*	20
A. kretschmeri	20	*A. vallsii*	20
A. rigonii	20	*A. subcoriacea*	20
A. chiquitana	20	*A. hassleri*	20
A. matiensis	20	*A. pflugeae*	20
Section *Rhizomatosae*			
A. burkartii	20	*A. glabrata* var. *glabrata*	40
A. pseudovillosa	40	*A. glabrata* var. *hagenbeckii*	40
A. nitida	40		
Section *Arachis*			
A. glandulifera	20	*A. decora*	18
A. cruziana	20	*A. herzogii*	20
A. monticola	40	*A. microsperma*	20
A. magna	20	*A. villosa*	20
A. ipaënsis	20	*A. helodes*	20
A. valida	20	*A. correntina*	20
A. williamsii	20	*A. simpsonii*	20
A. batizocoi	20	*A. cardenasii*	20
A. duranensis	20	*A. kempff-mercadoi*	20
A. hoehnei	20	*A. diogoi*	20
A. stenosperma	20	*A. kuhlmannii*	20
A. praecox	18	*A. gregoryi*	20
A. palustris	18	*A. krapovickasii*	20
A. benensis	20	*A. linearifolia*	20
A. trinitensis	20	*A. schininii*	20
		A. hypogaea	40

A. monticola and *A. hypogaea* are, therefore, tetraploids, which have attained diplontic behavior, though they sometimes show secondary associations in the form of quadrivalents and trivalents (Singh and Moss 1982). In addition to the basic chromosome number $n = 10$ found in most of the diploid and tetraploid species in all the nine sections of the genus *Arachis*, the basic chromosome number $n = 9$ has also been found in four diploid species, *A. palustris* (Lavia 1996), *A. praecox* (Lavia 1998), and *A. decora* (Penaloza et al. 1996) of section *Arachis* and *A. porphyrocalyx* of section *Erectoides* (Penaloza and Valls 2005). It has been suggested that the $n = 9$ constitutes a derived number from $n = 10$ (Lavia 1998); however, the cytogenetic mechanism involved in its origin is not yet known with certainty.

Smartt et al. (1978a) identified two genomes (A and B) in section *Arachis*, both of which occur in cultivated groundnut (*A. hypogaea*) and the tetraploid wild species *A. monticola*, which was further supported by later studies in the two-genome theory in cultivated groundnut, following the chromosome analysis (Stalker and Dalmacio 1981; Singh and Moss 1982). Subsequently, using chromosome morphology and crossing relationships, three genomes (A, B and D) were proposed in section *Arachis* diploid species (Smartt 1965; Smartt et al. 1978a; Singh and Moss 1982, 1984a, b; Stalker 1991). The A-genome is characterised by a pair of chromosomes smaller than the other chromosomes, while the B-genome lacks this smaller chromosome pair. Most diploid wild species contain the A-genome. Only a single B-genome species *A. batizocoi* was initially recognized, but now several others have been identified (Fernandez and Krapovickas 1994). The only D-genome diploid species is *A. glandulifera*, native to eastern Bolivia (Stalker 1991). "A"-genome species show considerable variation in fertility levels among the progenies from the crosses within A-genome species (Smartt 1965; Gregory and Gregory 1979; Spielman et al. 1979; Stalker and Wynne 1979; Singh and Moss 1982, 1984a). Subsequently, using the crossing relationships that Gregory and Gregory (1979) initiated, Smartt and Stalker (1982) proposed a series of genomes for diploid species in the genus *Arachis*, which include the following:

A = section *Arachis*, perennials and most annuals
B = section *Arachis* (*A. batizocoi*)
D = section *Arachis* (*A. glandulifera*)

Am = section *Ambinervosae*
C = section *Caulorrhizae*
E = section *Erectoides* (subgenomes E_1, E_2, E_3, corresponding to series)
Ex = section *Extranervosae*
T = section *Triseminatae*
R_1 = section *Rhizomatosae*, series Prorhizomatosae

The nuclear DNA content has an important function in the evolution and adaptation of the plants (Price 1976; Bennett 1982). Lavia and Fernández (2008) studied the genome size of 16 species of *Arachis* with $n = 10$ and three with $n = 9$, involving both diploid and tetraploid species. DNA content (2C) between all diploid species of *Arachis* with $2n = 20$ varied from 2.87 pg in *A. retusa* to 6.59 pg in *A. douradiana*. Likewise, the DNA content in species with $2n = 18$ varied from 3.26 pg in *A. palustris* to 4.16 pg in *A. decora*. The species with greater DNA contents have the longest chromosomes, while those with lower DNA contents have smaller chromosomes. In contrast, DNA content in *A. hypogaea* ($2n = 40$) ranged between 10.87 and 11.92 pg. These results suggest that in the evolution of *Arachis* genome, both increases and diminution of DNA content would have occurred. Species with greater DNA content are included in sections believed to have a more recent origin, whereas those that contain lower DNA content belong to the oldest section, suggesting genome evolution of *Arachis* towards higher DNA content. Reduction of the DNA content after polyploidization would have happened in *A. hypogaea* (Lavia and Fernández 2008).

1.2.5 *Crossing Relationships Among Wild* Arachis *Species*

Gregory and Gregory (1979) reported successful intrasectional hybrids in sections *Arachis*, *Erectoides*, *Rhizomatosae*, *Caulorrhizae*, *Extranervosae*, *Triseminatae* and *Ambinervosae* and the intersectional hybrids involving *Arachis* with *Erectoides* and *Rhizomatosae*; *Erectoides* with *Rhizomatosae*, *Caulorrhizae* and *Ambinervosae* and *Ambinervosae* with *Extranervosae*, which led to the establishment of intra- and intersectional crossing relationships between the nine sections of the genus *Arachis* (Krapovickas and Gregory 1994). No successful intersectional cross of the diploid annual wild *Arachis* species belonging to section *Arachis* were obtained with those in section *Triseminatae*, *Rhizomatosae* (*A. burkartii*), and with perennials and tetraploid species of the section *Arachis* (Krapovickas and Gregory 1994). A very high level of genetic isolation was found among the sections *Erectoides*, *Trierectoides*, *Extranervosae*, *Triseminatae* and *Heteranthae*, confirming their primitiveness in the genus *Arachis*, which has been further supported by the comparative morphology of the "B" (SAT) chromosome and the absence of the "A" pair (Fernandez and Krapovickas 1994). On the basis of taxonomic and cross-compatibility studies, Krapovickas and Gregory (1994) suggested that *Trierectoides*, *Erectoides*, *Extranervosae*, *Triseminatae* and *Heteranthae* are the oldest sections while *Procumbentes*, *Caulorrhizae*, *Rhizomatosae* and *Arachis* are of more recent origin. Intersectional hybrids involving section *Arachis* with *Rhizomatosae*, *Extranervosae*, *Procumbentes* and *Erectoides* have also been successful at ICRISAT (Mallikarjuna and Bramel 2001; Mallikarjuna 2002, 2005).

1.3 Taxonomy and Species Diversity of Wild *Arachis* Species

Krapovickas and Gregory (1994) used 32 descriptors, mostly morphological traits, to study taxonomy of 69 *Arachis* species. Using taxonomy and crossing incompatibility studies, they classified 69 species to nine sections and suggested that *Trierectoides*, *Erectoides*, *Extranervosae*, *Triseminatae* and *Heteranthae* are the oldest sections while *Procumbentes*, *Caulorrhizae*, *Rhizomatosae* and *Arachis* are of more recent origin. Valls and Simpson (2005) described 11 new species (*A. porphyrocalyx*, *A. submarginata*, *A. pflugeae*, *A. hassleri*, *A. interrupta*, *A. seridoënsis*, *A. nitida*, *A. linearifolia*, *A. shcininii*, *A. gregoryi* and *A. krapovickasii*) of *Arachis*, representing seven of the nine taxonomic sections of the genus. Of these, eight were earlier classified in Krapovickas and Gregory (1994) monograph, but are now treated with their own specific epithet. Thus, the description of these 11 species will help clarify the systematics of the genus *Arachis*, as well as aid in understanding the evolutionary pathway of certain important materials. Some of these may have played a role in developments that led to the origin of cultivated groundnut. The key morphological

features that distinguish these species include growth habit (procumbent, erect, prostrate and decumbent), types of leaves (trifoliate and tetrafoliate), plant type (rhizomatous and nonrhizomatous), leaflet shape, leaflet surface, leaflet length and width, petiole length, leaflet margins, presence or absence of bristles on stipules, petiole and leaflet surface, standard petal and wing color and stem and peg characteristics (Krapovickas and Gregory 1994, 2007; Valls and Simpson 2005). Further, the species in sections *Arachis* and *Rhizomatosae* are characterized by short pegs that grow vertically in comparison to the species in the other seven sections in which the pegs are very long and superficial.

A. Pintoi is a herbaceous perennial species grown for multipurpose use, ranging from use as forage, ground cover in fruit orchards, forest and low tillage system, erosion control, and ornamental purposes. Carvalho and Quesenberry (2009) characterized *A. Pintoi* accessions for phenotypic diversity, which represented great morphological variability. Of the 595 correlations computed, 96 were statistically significant. They detected biologically meaningful correlations ($r^2 = 0.50$) for leaf length and pod weight, leaf length and pod width, leaf length and seed weight and leaf length and seed width. Total genetic diversity in this study was 0.71, with both principal component and cluster analysis differentiating the accessions into four distinct groups. Researchers at ICRISAT have characterized 267 wild *Arachis* accessions of 37 species for 33 qualitative and 15 quantitative traits (Table 1.3) at *Arachis* house, wherein six plants of each of the 267 accessions were grown under large-size cylindrical concrete structures. Preliminary results revealed that species exhibited large variation for lateral branches, plant width, stipule length, adnation of stipule on the main stem, petiole length on the main stem, apical leaflet length and width on the main stem, apical length and width on the primary lateral, hypanthium length, standard petal length and peg length, with Shannon-Weaver diversity index ranging from 0.022 for hairiness on the margin of the stipule of the main stem to 0.836 for basal leaflet shape on the primary lateral (Upadhyaya unpublished data).

Unlike cultivated groundnut germplasm, the evaluation of wild relatives in the field is not feasible because of their long generation time (from annual to perennial life cycle), extensive ground coverage, and thus the chance of mixing with other accessions, and risk of leftover pods/seeds remaining deep in the soil after harvest, thus becoming a source of contamination for the next crop. Researchers have, therefore, used isozyme and hybridization- and PCR-based markers to assess the intra- and interspecific variation, which have revealed high variability among wild *Arachis* species. The variability and relationship among 15 accessions of *A. glabrata* were studied by using isozymes (Maass and Ocampo 1995). In this study, polyacrylamide gel electrophoresis (PAGE) was applied to rhizome-tip tissue, which showed a high degree of intraspecific polymorphism for the isozymes α-EST, ACP, GOT and DIA. The four isozyme systems differentiated all the 15 accessions of *A. glabrata*. Using restriction fragment length polymorphism (RFLP) markers, Gimenes et al. (2002) analyzed four A-genome species (*A. cardenasii*, *A. correntina*, *A. duranensis*, *A. kempff-mercadoi*), three B-genome species (*A. batizocoi*, *A. ipaënsis*, *A. magna*), the AABB allotetraploid *A. hypogaea* and introgression lines resulting from a cross between *A. hypogaea* and *A. cardenasii*. All the *A. batizocoi* accessions were clustered in a separate group, suggesting that this species is not closely related to *A. hypogaea*, *A. ipaënsis* or the A-genome species analyzed. The highest level of genetic variation was found in *A. cardenasii* indicating that all accessions of wild species of *Arachis* might not be autogamous, as reported for *A. hypogaea* (Gimenes et al. 2002). Nóbile et al. (2004) evaluated genetic variability within and among accessions of wild *Arachis* species, *A. glabrata*, *A. burkartii*, *A. pseudovillosa* and *A. nitida* belonging to the section *Rhizomatosae* using *random amplified polymorphic DNA* (*RAPD*) markers that detected the highest genetic variation in diploid species *A. burkartii*. The diploid species *A. burkartii* and the tetraploid species *A. glabrata*, *A. pseudovillosa* and *A. nitida* were grouped separately, suggesting that none of these tetraploid species originated from *A. burkartii*. Hoshino et al. (2006) used heterologous simple sequence repeat (SSR) markers to characterize genetic diversity among 76 accessions of 34 species from nine sections of the genus *Arachis*. The total number of alleles ranged from 28 in *A. tuberosa* (section *Trierectoides*) to 81 in *A. paraguariensis* (section *Erectoides*). All the species investigated showed high polymorphism among their accessions; however, accessions were not grouped exactly according to the species and sections to which they belonged. This difference may be attributed to the high polymorphism

Table 1.3 List of descriptors used for characterizing wild *Arachis* species accessions at ICRISAT, Patancheru, India

Qualitative descriptor
Growth habit (GH); root growth (RG); stem modification (SM: absent, rhizome and stolon); branching pattern (BP: alternate 2:2:2, alternate 2:1:2 or 1:2:1, sequential, irregular); pigmentation on main stem (PMS: absent, present), main stem hairiness [MSH: Glabrous, Subglabrous (hairs in one or two rows), Moderately hairy (hairs in 3–4 rows), Very hairy (stem surface mostly covered with hairs), wooly (villous hairs >2 mm)]; main stem hair type [MSHT: Glandular (bristles), Non-glandular, or both types]; flowers on main stem (FLM); hypanthium hairiness [HYH: Glabrous, Hairy, wooly (villous hairs >2 mm)]; standard petal color [SPC: White (155 A–D), Lemon yellow (6 A–B), Yellow (14 A–B), Orange-yellow/yellow-orange (24 B), Orange (24 A or 25 A), Dark orange (28 A), Garnet/brick red/reddish orange (35 A)]; standard petal markings on front face [SPMFF: Absent, Lemon yellow (6 A–B), Yellow (14 A–B), Orange-yellow/yellow-orange (24 B), Orange (24 A or 25 A), Dark orange (28 A), Garnet/ brick red/reddish orange (35 A), Purple]; standard petal markings on back face (SPMBF: Absent, Red or purple blush, Red or purple streaks, Grayed orange streaks, and Greenish purple streaks); wing petal color (WPC: Yellow, lemon yellow, orange, yellow and white); leaflet surface on main stem (LSMS: Non-shiny, shiny); leaflet surface on primary lateral (LSPL: Non-shiny, shiny); leaflet color (LC) on main stem and on primary laterals [Yellow/yellow-green (146 A–D), light green (137 A–D), green (139 A–B), dark green (131 A), bluish green and purplish green]; leaflet shape on apical main stem (LSAMS: Cuneate, obcuneate, wide-elliptic, narrow-elliptic, elliptic, suborbicular, orbicular, ovate, obovate, oblong, oblong-lanceolate, lanceolate, ob-lanceolate, linear-lanceolate, others); leaflet shape on apical primary lateral (LSAPL: same as described for LSAMS); leaflet shape on basal main stem (LSBMS: same as described for LSAMS); leaflet shape on basal primary lateral (LSBPL: same as described for LSAMS); leaflet hairiness (LH) on main stem [Glabrous; almost glabrous on both surfaces; almost glabrous above, hairy below; almost glabrous below, hairy above; hairy on both surfaces; wooly(villous hairs >2 mm)]; leaflet hairiness on primary lateral (LHPL: same as described for LH); leaflet bristle (LB) on main stem (Absent, bristles on upper surface, bristles on lower surface, bristles on both surface); leaflet bristle on primary lateral (LBPL: Absent, bristles on upper surface, bristles on lower surface, bristles on both surface); leaflet hairiness on margin of main stem and primary laterals [LHMSPL: Absent, ciliate, wooly (villous hairs >2 mm)]; leaflet bristle on margin of main stem and primary laterals (LBMSPL: Absent, few, setose); leaflet midrib hairiness on upper main stem (LMHUMS: Glabrous, subglabrous, hairy); leaflet midrib hairiness on upper primary lateral (LMHUPL: Glabrous, subglabrous, hairy); leaflet midrib hairiness on lower main stem (LMHLMS: Glabrous, subglabrous, hairy); leaflet midrib hairiness on lower primary lateral (LMHLPL: Glabrous, subglabrous, hairy); leaflet tip shape of the main stem (LTSMS: Acuminate, acute, indented, mucronate, obtuse); leaflet tip shape of primary lateral (LTSPL: Glabrous, subglabrous, hairy); nature of stipule on primary lateral (NSTPL: Open, partially open, tubular); stipule hairiness on outside of the main stem [SHOMS: Glabrous, Subglabrous, Hairy, Very hairy, wooly (villous hairs >2 mm)]; stipule hairiness on outside primary lateral [SHOPL: Glabrous, Subglabrous, Hairy, Very hairy, wooly (villous hairs > 2mm)]; stipule hairiness on margin of the main stem [SHMMS: Glabrous, Subglabrous, Hairy, Very hairy, wooly (villous hairs >2 mm)]; stipule hairiness on margin of primary lateral branch [SHMPLB: Glabrous, Subglabrous, Hairy, Very hairy, wooly (villous hairs >2 mm)]; stipule bristles outside of the main stem (SBOMS: absent, a few, many); stipule bristle outside primary lateral branch (SBOPLB: absent, a few, many); stipule bristles on margin of the main Stem (SBMMS: absent, a few, many); stipule bristle on the margin of primary lateral (SBMPL: absent, a few, many); nature of petiole on main stem and on primary laterals (NPMSPL: Straight, slightly reflexed, reflexed); petiole hairiness on main stem [PHMS: Glabrous, subglabrous, hairy, very hairy, wooly (villous hairs >2 mm)]; petiole hairiness on primary lateral [PHPL: Glabrous, subglabrous, hairy, very hairy, wooly (villous hairs >2 mm)]; petiole bristle on main stem (PBMS: Absent, few, many); petiole bristle on primary lateral (PBPL: Absent, few, many); petiole groove on main Stem [PGMS: Absent (0%), shallow (<15%), deep (16–30%), very deep (>30%)]; petiole groove on primary lateral [PGPL: Absent (0%), shallow (<15%), deep (16–30%), very deep (>30%)]; rachis groove on main stem [RGM: Absent (0%), shallow (<15%), deep (16–30%), very deep (>30%)]; rachis groove on primary lateral [RGPL: Absent (0%), shallow (<15%), deep (16–30%), very deep (>30%)]; peg growth (PG: Almost horizontal, almost vertical, twisted); peg pigmentation (PGP: absent, present); pod beak (PB: Absent, slight, moderate, prominent, very prominent); pod reticulation (PR: Smooth, slight, moderate, prominent, very prominent); seed color [SC: Off-white (158 A–D and 159 C–D), tan (173 C–D and 174 C–D)]; number of segments between pods (NSBP: 1 segment, 2 segments, 1–2/2–1 segments, 1–2–3 segments, 2–3 segments, 3 segments)
Quantitative descriptor
Days to emergence (DE); days to 50% flowering (DF); upper lip calyx lobation [ULCL: indentation and number of lobes of the upper lip of calyx of flowers recorded in 4 classes (entire, 2 lobes, 3 lobes, 4 lobes) 4–6 months after emergence], number of lateral branches (NLB); standard petal length (SPL); standard petal width (SPW); leaflet length of the apical primary lateral (LLAPL); leaflet width of apical main stem (LWAMS); leaflet width of apical primary lateral (LWAPL); leaflet length on basal main stem (LLBMS); leaflet length on basal primary lateral (LLBPL); leaflet width on basal main stem (LWBMS); leaflet width on basal primary lateral (LWBPL); stipule length on main stem (SLMS); stipule length on primary lateral branch (SLPLB); stipule adnation length on main stem (SALMS: measured as length of adnate part of stipule of fourth leaf); stipule adnation length on primary lateral branch (SALPLB); stipule adnation width on main stem (SAWMS); stipule adnation width on primary lateral branch (SAWPLB); petiole length on main stem (PLMS); petiole length of primary lateral (PLPL); rachis length of the main stem (RLMS); rachis length of primary lateral (RLPL); main stem height (MSH); main stem thickness (MST); days to maturity (DM), peg length (PL); basal segment length (BSL); apical segment length (ASL); pod length (PDL); pod width (PDW), length of first isthmus (LFI); seed length (SDL); seed width (SDW), 100 seed weight (HSW)

found in some of the loci and sharing of alleles among species from different sections. Gimenes et al. (2007) reported high transferability of microsatellite markers of *A. hypogaea* to other species of the genus, and identified two groups – the first consisting of *A. hypogaea*, *A. monticola* and all the analyzed A-genome species while the second contained B- and D-genome species. Mallikarjuna et al. (2007) studied the genetic relationship among two *A. diogoi* accessions and three *A. chiquitana* accessions, using SSRs and high-throughput assay. Two *A. diogoi* accessions, ICG 4983 and ICG 8962, and the two *A. chiquitana* accessions, ICG 13181 and ICG 13241, formed two distinct groups. The third *A. chiquitana* accession, ICG 11560, did not group closely with the other *A. chiquitana* accessions, but showed a closer relationship with them than with the *A. diogoi* accessions. These results showed that *A. chiquitana* accessions, particularly ICG 11560, are not related to the accessions of *A. diogoi* and that the accessions belonging to these two species are different.

Angelici et al. (2008) used SSRs to study the genetic diversity among 77 accessions of the four species from section Rhizomatosae, the diploid *A. burkartii* and the tetraploid *A. glabrata*, *A. pseudovillosa* and *A. nitida*. The 15 SSR loci detected 249 alleles and high degrees of intra- and interspecific polymorphism. The diploid accessions grouped in one cluster and the tetraploid accessions in another cluster. The markers differentiated all the 77 accessions but the genetic distance could not be correlated with geographic origin. Furthermore, Robledo and Seijo (2008) studied the genomic affinities of *A. glandulifera* with A- and B-genome by comparing several chromosome landmarks and by total genome hybridization, using fluorescence in situ hybridization (FISH) of the 5S and 45S rRNA genes and heterochromatic 4′-6′-diamidino-2-phenylindole (DAPI) positive bands. Their results revealed very poor homologies with all the A- and B-genome taxa, supporting the special genome constitution (D-genome) of *A. glandulifera*. In a study involving 14 wild *Arachis* species from different sections and 24 allotetraploid groundnut cultivars from several countries and belonging to different botanical types, Tang et al. (2008) revealed that groundnut cultivars were closely related to each other, and shared a large number of alleles. In contrast, the species in genus *Arachis* shared few alleles. Further, the cultivars in this study could be partitioned into two main groups and four subgroups at the molecular level, and that *A. duranensis* is one of the wild ancestors of *A. hypogaea*. The lowest genetic variation was detected between *A. cardenasii* and *A. batizocoi*, and the highest between *A. pintoi* and the species in the section *Arachis*. This study also revealed that accessions in section *Heteranthae* were closest to the tested accessions in section *Arachis*, followed by the tested accessions in the sections *Procumbentes*, *Rhizomatosae* and *Caulorrhizae*, respectively, thus providing breeders insights into the use of wild species out of section *Arachis*, for the improvement of cultivated groundnut. At ICRISAT, 47 accessions of 14 wild *Arachis* species along with 805 accessions of cultivated *A. hypogaea* (322 accessions of *hypogaea* type and 483 of *fastigiata* type) were genotyped, using 21 SSR markers. The common alleles were higher in the wild *Arachis* species (359) than in the cultivated *fastigiata* (230) and *hypogaea* (190) types. Wild species also possessed the highest number of unique alleles (101), and the gene diversity was 0.870, ranging from 0.434 to 0.947. The wild *Arachis* accessions shared only 15 alleles with the subspecies *hypogaea* and 32 alleles with the subspecies *fastigiata*.

1.4 Wild *Arachis* as Source of Variation for Agronomic Traits

Wild *Arachis* species harbor very high levels of resistance to many biotic and abiotic stresses when compared with cultivated groundnut (Dwivedi et al. 2003, 2008; Table 1.4). Examples include resistance to rust, early leaf spot, late leaf spot, nematode, peanut mottle virus, peanut stripe virus, peanut bud necrosis virus, tomato-spotted wilt virus, groundnut rosette disease, aflatoxin, corn ear worm, southern corn root worm, thrips, leaf hoppers and *Spodoptera*. Further, the mechanism and genetic control of resistance in wild relatives appear to be different than that in cultivated types. For example, resistance to rust in crosses involving wild relatives is partially dominant (Singh et al. 1984). Sharma et al. (2003) found several morphological traits associated with tolerance to insect pests. For example, main stem thickness and hairiness, hypanthium length, leaflet shape and length, leaf hairiness, standard petal length and petal markings, basal leaflet width, stipule adnation length and width, and peg length showed significant correlation with damage by

Table 1.4 Wild *Arachis* species resistant to pests and diseases

Species	Trait	References
A. hagenbeckii, *A. glabrata* and *A. repens*	Early leaf spot (ELS)	Gibbons and Bailey (1967)
A. diogoi and *A. cardenasii*	ELS and late leaf spot (LLS)	Abdou et al. (1974)
A. glabrata	Peanut mottle virus (PMV)	Demski and Sowell (1981)
A. chacoense, *A. cardenasii*, *A. stenosperma*, *A. repens*, *A. appressipila*, *A. paraguariensis*, *A. villosulicarpa*, *A. hagenbeckii*, *A. glabrata*, *A. batizocoi*, *A. duranensis*, *A. correntina*, *A. villosa* and *A. pusilla*	LLS, rust	Subrahmanyam et al. (1983, 1985a)
A. pusilla, *A. cardenasii*, *A. diogoi* and *A. correntina*	PMV, tomato-spotted wilt virus (TSWV)	Subrahmanyam et al. (1985b)
A. monticola	ELS	Subrahmanyam et al. (1985c)
A. batizocoi and *A. cardenasii*	Nematode	Nelson et al. (1989) and Holbrook and Noe (1990)
A. cardenasii, *A. chacoense* and *A. stenosperma*	ELS, LLS, rust	Nigam et al. (1991)
A. cardenasii and *A. duranensis*	Seed colonization and aflatoxin production	Nigam et al. (1991)
A. chacoense and *A. pusilla*	PMV	Nigam et al. (1991)
A. cardenasii	Peanut stripe virus (PStV)	Nigam et al. (1991)
A. correntina, *A. chacoense*, *A. stenosperma* and *A. villosulicarpa*	Insect-pests	Nigam et al. (1991)
A. chacoense	ELS, TSWV, rust, nematode, thrips, corn earworm (CEW), leaf hoppers	Stalker (1992)
A. cardenasii, *A. stenosperma* and *A. batizocoi*	LLS, TSWV, ELS, rust, nematode, CEW, leaf hoppers	Stalker (1992)
A. helodes, *A. sylvestris*, *A. kretschmeri*, *A. kuhlmannii* and *A. stenosperma*	Nematode	Sharma et al. (1999)
A. benensis, *A. cardenasii*, *A. villosa*, *A. appressipila* and *A. triseminata*	Peanut bud necrosis virus	Reddy et al. (2000)
A. appressipila, *A. triseminata*, *A. magna*, *A. sylvestris*, *A. pusilla*, *A. valida* and *A. dardani*	ELS	ICRISAT (2000)
A. hoehnei, *A. duranensis* and *A. kuhlmannii*	LLS, rust	Pande and Rao (2001)
A. diogoi, *A. hoehnei*, *A. kretschmeri*, *A. appressipila*, *A. cardenasii*, *A. villosa*, *A. stenosperma*, *A. pintoi*, *A. kuhlmannii*, *A. triseminata* and *A. decora*	Groundnut rosette disease	Subrahmanyam et al. (2001)
A. cardenasii	Rust, ELS, nematode, southern corn rootworm, leaf hopper	Stalker et al. (2002a, b) and Stalker and Lynch (2002)
A. cardenasii, *A. duranensis*, *A. kempff-mercadoi*, *A. monticola*, *A. stenosperma*, *A. paraguariensis*, *A. pusilla* and *A. triseminata*	Leaf miner, *Helicoverpa*, leaf hopper, rust, LLS	Sharma et al. (2003)
A. kempff-mercadoi	ELS, LLS, *Spodoptera*	Mallikarjuna et al. (2004)

Helicoverpa armigera, *Spodoptera litura* and leafhoppers. Wild relatives are also reported to possess high oil and protein content (Dwivedi et al. 2003). Several *Arachis* species are extremely drought tolerant (Stalker and Moss 1987). At ICRISAT, 282 wild *Arachis* accessions belonging to 38 species were evaluated for soil plant analysis development (SPAD), chlorophyll meter readings (SCMR) and specific leaf area (SLA) traits related to drought tolerance at two stages, viz., 60 days after sowing (DAS) and 80 DAS. Enormous variability was observed among the accessions for these two traits, which ranged from 26.41 to 62.38 for SCMR at 60 DAS and 29.01 to 60.28 at 80 DAS, 39.23 to 357.70 for SLA at 60 DAS and 91.53 to 209.39 at 80 DAS (Upadhyaya unpublished data). More recently, Nautiyal et al. (2008) reported wide genetic variability in leaf characteristics such as color, shape, hairiness, specific leaf area (SLA, length, width and thickness) among wild *Arachis* species, that were associated with cold and heat tolerance as measured by relative leaf

injury (RI). The SLA in 36 wild *Arachis* accessions ranged from 66 to 161 $cm^2\ g^{-1}$. Using RI as the measure of tolerance, *A. glabrata* 11,824 and *A. paraguariensis* 12,042 were identified as heat and cold tolerant, respectively, while *A. appresipila* 11,786 was found to be susceptible to both heat and cold stress. Further, when detecting the concentration of various leaf constituents, the total protein, phenols, sugars, reducing sugar, amino acids, proline, epicuticular wax load, and chlorophyll were found to vary significantly among heat- and cold-tolerant accessions. For example, the epicuticular wax load ranged between 1.1 and 2.5 mg dm^2 among 13 *A. glabrata* accessions. The high-wax accessions showed a higher diffusion resistance (dr) as compared to low-wax type; though the transpiration rate (tr) in high-wax type was moderate (between 9.5 and 11.6 $\mu g\ cm^{-2}\ s^{-1}$). These accessions also showed large genetic variation in canopy temperature as well. For example, the fully turgid leaves with relative water content $\geq$ 91% showed leaf water potential (ψ_{leaf}) between -0.7 and -1.2 MPa. These results revealed that plants with thicker leaves are better protected from heat injuries while epicuticular wax load helps in maintaining stomotal regulation and leaf water relations, thus affording adaptation to wild *Arachis* species to thrive under water-limited environments. The genetic upgradation of the cultivated groundnut necessitates the use of wild *Arachis* gene pools to expand its genetic variability.

1.5 Barriers to Interspecific Hybridization

Many of the wild *Arachis* species are not cross-compatible with cultivated groundnut. The major barrier for gene introgression is postzygotic failure of embryo development. Researchers have used a number of techniques to either circumvent or overcome barriers to hybridization, which include the use of hormonal treatment to overcome pre- and postfertilization barriers or embryo rescue, if postfertilization barriers exist.

The species in the secondary gene pool, which is represented by the diploid species of the section Arachis, have greater potential as they possess very high levels of resistance to many pests and diseases. However, utilization of the secondary gene pool for the introgression of useful genes into *A. hypogaea* shows sterility barriers due to different ploidy levels, genomic incompatibilities and cryptic genetic differences, which could be restored by manipulating ploidy levels, as discussed in Sect. 1.10.

Direct intersectional hybridization with *A. hypogaea* has been difficult, necessitating the use of hormonal treatment to overcome pre- and postfertilization barriers or embryo rescue, if postfertilization barriers exist, which may, to a large extent, be overcome using in vitro techniques such as cell and protoplast culture, ovule and embryo culture or both. For example, in vitro culture of ovules or embryos has been successfully used to produce intersectional hybrids in many genera (Narayanaswami and Norstog 1964; Collins et al. 1984). However, several factors including genotypic specificity, media composition, concentrations of growth hormones and environmental conditions alone or in combinations influence the successful use of ovule and embryo culture techniques in interspecific crosses particularly with species from more distant gene pool (Sastri and Moss 1982; Mallikarjuna and Sastri 1985a, b). The intersectional hybrid between *A. hypogaea* and *A. glabrata* has been developed following embryo rescue technique and the resultant hybrid inherited the resistance to rust, late leaf spot, peanut bud necerosis and peanut stripe diseases from the pollen parent *A. glabrata* (Mallikarjuna 2002; Mallikarjuna and Sastri 2002). Likewise, using hormonal application to the pollinated pistil followed by embryo rescue technique, Mallikarjuna (2005) produced the first fertile intersectional hybrid between *A. hypogaea* and *A. chiquitana* of section *Procumbentes*. *A. chiquitana* is reported resistant to seed colonisation by *Aspergillus flavus*. Clearly, these studies demonstrate that it is possible to access the desirable traits across the sections for broadening the genetic base of cultivated groundnut by following various approaches.

1.6 Genomic Resources to Monitor Introgression in Interspecific Crosses Involving Wild *Arachis* Species

The genetic linkage maps based on interspecific crosses will be useful in locating specific genes of interest in the interspecific progenies that provide

a way to accomplish interspecific gene transfer with minimum linkage drag, thus improving the prospects for successful introgression of desirable genes from wild relatives (Tanksley et al. 1989; Tanksley and McCouch 1997). Halward et al. (1993) were the first to report RFLP-based genetic linkage map, involving 87 F_2 population of the cross involving diploid *Arachis* species *A. stenosperma* and *A. cardenasii*, with a total map distance of 1,063 cm, which contained 117 RFLP loci on 11 linkage groups. Burow et al. (2001) used BC_1F_1 population (78) derived from synthetic amphiploid TxAG-6 (Simpson et al. 1993) and Florunner to develop the first RFLP-based tetraploid genetic map, which mapped 370 RFLP loci on 23 linkage groups (LGs) with a total map distance of 2,210 cm. Subsequently, Moretzsohn et al. (2005) constructed the first SSR-based genetic map of *Arachis* by using F_2 population, involving AA-genome diploid species *A. duranensis* and *A. stenosperma*, which mapped 170 SSR loci on 11 LGs covering 1,231 cm and average marker density of 7.24 cm. Gobbi et al. (2006) mapped 130 SSR loci on 10 LGs involving diploid B-genome donor species, *A. ipaënsis* and *A. magna*. It is expected that with the availability of AA- and BB-genome-based genetic maps for *Arachis*, it would be possible to use these segregating loci to tag gene of interest in interspecific crosses. The group in Brazil now uses synthetic amphidiploids to construct a reference map, which they are using to access the near-complete genome sequences of model legumes (*Medicago truncatula* and *Lotus japonicus*), in a way that would enhance understanding of the *Arachis* genome. To do that, they placed more than 80 legume anchor markers (Fredslund et al. 2006) on the AA-genome map and analyzed the synteny between *Arachis* and the model legumes, identifying affinities in nine of the ten *Arachis* linkage groups and model legume chromosomes, some showing substantial regions of marker colinearity (Moretzsohn et al. 2007). The combination of SSR-based genetic maps of diploid species and synthetic amphiploids incorporating various exotic genomes would unlock the hidden treasure in wild *Arachis* species and would facilitate the marker-assisted introgression of important traits into the cultivated groundnut.

Guimarães et al. (2008) constructed and characterized two large-insert bacterial artificial chromosome (BAC) libraries, one for each of the diploid ancestral species. The libraries (AA and BB) are ca. 7.4 and ca. 5.3 genome equivalents, respectively, with low organelle contamination and average insert sizes of 110 and 100 kb. These diploid BAC libraries are important tools for the isolation of wild alleles conferring resistances to biotic stresses, comparisons of orthologous regions of the AA and BB-genomes with each other and with other legume species and will facilitate the construction of a physical map.

Garcia et al. (1995) showed introgression of genes from *A. cardenasii* into *A. hypogaea* in 10 of 11 LGs, which they used to enhance the selection efficiency for developing nematode-resistant germplasm (Garcia et al. 1996). Burow et al. (1996) identified two random amplified polymorphic DNA (RAPD) markers linked with nematode resistance in BC_4F_2 population of the cross Florunner × TxAg-6 that were closely linked to each other. One marker RKN229 was 9 cm away from resistance locus (Burow et al. 1996). Two dominant genes that conferred resistance on root-knot nematode, *Meloidogyne arenaria* race 1, were mapped using RAPD and sequence characterized amplified region (SCAR) markers (Garcia et al. 1996). A marker Z3/265, closely linked with nematode resistance, was mapped to a linkage group in a backcross population known to contain *A. cardenasii* introgression (Garcia et al. 1996), which they cloned to make SCAR and RFLP probes that further confirmed the linkage with nematode resistance. The RFLP markers linked to a locus for resistance to *M. arenaria* race 1 has been identified by various workers using mapping populations derived from interspecific crosses (Choi et al. 1999; Church et al. 2000; Seib et al. 2003), thus providing a useful selection method for identifying resistance to the peanut root-knot nematode.

1.7 Approaches to Interspecific Gene Transfer

The differences in ploidy levels have been the major bottleneck in interspecific gene transfer between diploid wild *Arachis* species and tetraploid *A. hypogaea*. For the successful utilization of wild *Arachis* species in the genetic amelioration of the cultivated groundnut cultivars, Simpson (2001) has outlined the following approaches to overcome the genomic imbalances in crosses involving species with different ploidy levels.

1.7.1 Hexaploid Route

A. hypogaea ($2n = 4x = 40$) is hybridized with a diploid wild *Arachis* species ($2n = 2x = 20$) to produce a sterile triploid ($2n = 3x = 30$), which is then treated with colchicine to produce hexaploid ($2n = 6x = 60$). This amphiploid is first crossed and then selfed or backcrossed with *A. hypogaea* until the tetraploid hybrid is obtained after eliminating the excess chromosomes during segregation. This pathway has been used with some success in North Carolina State University, Raleigh, USA, and at ICRISAT for developing numerous disease- and insect-resistant elite germplasm. This technique could be used with several variations such as crossing two or more diploid species before crossing with *A. hypogaea*. This approach has limitation, as it is time consuming and unpredictable. However, the advantage is through selfing as selfing the amphiploids increases recombination between the chromosomes of different genomes.

1.7.2 Diploid/Tetraploid Pathway using Bridge Species

This pathway has been the most successful introgression pathway at the Texas A&M University, USA, for gene transfer from wild *Arachis* species into *A. hypogaea* (Simpson 1991; Simpson and Starr 2001), using B-genome species as a bridge species. *A. cardenasii* was first crossed with *A. diogoi*, both diploid species and the resulting hybrid (52% pollen stained) was crossed as male parent with *A. batizocoi*, the "B"-genome diploid species. The resulting diploid three-way hybrid was sterile (pollen stained $<1\%$) and was subsequently treated with colchicines. The amphiploid ($> 90\%$ pollen stained) was easily crossed with *A. hypogaea* cv. Florunner. Selection was made for highly fertile-resistant progenies that were backcrossed to *A. hypogaea*. This approach had been proposed by Smartt et al. (1978b) as a solution to overcome the sterility barrier between *A. hypogaea* and the wild diploid species. They hypothesized that use of a B-genome parent might make the complex amphiploids more cross-compatible with *A. hypogaea*, but the B-genome species, *A. batizocoi*, is susceptible to late leaf spot and other diseases, which may lead to the incorporation of these unfavorable traits into the breeding lines, thus hampering the groundnut improvement programs (Holbrook and Stalker 2003).

1.7.3 Diploid/Tetraploid Pathway

The two diploid wild *Arachis* species are first doubled with colchicines, followed by the hybridization of these two amphidiploids to form a tetraploid hybrid, which is finally crossed with *A. hypogaea*, provided the amphiploid hybrid is fertile enough to make the cross. In order to transfer rust resistance from the wild *Arachis* species, autotetraploidy was induced in three diploid species, viz., *A. cardenasii*, *A. stenosperma* and *A. chacoense*, and the resultant autotetraploids were crossed with *A. hypogaea* cultivars. A number of *A. hypogaea*-like derivatives were identified with rust resistance transferred from wild species. Germplasm lines have not been released from this pathway in peanut till date. High level of sterility is the major factor that limits this technique.

Another variant of this pathway is to first cross two diploid *Arachis* species, double the chromosome number of the hybrid, and then cross the resultant amphidiploid with *A. hypogaea*. This pathway was attempted in Texas (Simpson 1991), but without both A- and B-genome types in the crossing scheme, the success is limited greatly because of high sterility factors.

1.8 Elite Germplasm Originating from Interspecific Crosses

To date, only species from primary and secondary gene pools have been exploited, leading to the development of many elite germplasm lines that originate from interspecific crosses, with resistance to rust, ELS, LLS, nematodes, southern corn rootworm, corn earworm, *Spodoptera*, and leaf hoppers were reported from interspecific crosses [reviewed in Dwivedi et al. (2003)]. However, these elite germplasm are good sources of resistance to many pests and diseases to enhance the levels of resistance in cultivated groundnut.

Spancross (Hammons 1970), Tamnut 74 (Simpson and Smith 1975), Coan (Simpson and Starr 2001), NemaTAM (Simpson et al. 2003), ICGV-SM 85048 (Nigam et al. 1998) and ICGV-SM86715 (Moss et al.

1998) were released for cultivation, mostly in the USA. Nematode resistance has helped US peanut growers to save US$100 million annually (*www.unep.org/documents/default.asp?documentID=399&article*). Likewise, the researchers at ICRISAT were able to improve the levels of resistance to rust and late leaf spot in newly developed breeding lines originating from crosses involving interspecific derivatives in the breeding program. Following interspecific hybridization, 16 breeding lines (ICGV 99001 to ICGV 99016) have been developed at ICRISAT, of which ICGV 99001 and 99004 are resistant to late leaf spot (LLS) and ICGV 99003 and 99005 are rust resistant (Singh et al. 2003).

1.9 New Approach to Interspecific Gene Transfer

Several attempts have been made to transfer variability from wild *Arachis* species into *A. hypogaea* using the methods described in Sect. 1.10. However, limited success has been realized from these approaches. Synthetic amphiploids have proved successful in generating new diversity in crops such as wheat and Brassicaceae [reviewed in Dwivedi et al. (2008)]. Using this approach, Simpson et al. (1993) crossed an AA-genome donor hybrid (*A. cardenasii* × *A. diogoi*) with a BB-genome species, *A. batizocoi*, and treated the resultant sterile hybrid with colchicine to double the chromosome number to obtain fertile hexaploid. This synthetic amphiploid, named TxAG-6, was subsequently crossed and backcrossed with the cultivated groundnut, which resulted in the release of two groundnut cultivars (Coan and NemaTAM) carrying genes for root-knot nematode (*M. arenaria*) resistance from *A. cardenasii* (Simpson and Starr 2001; Simpson et al. 2003). Another germplasm line, TxAG-7, was derived by crossing TP-129 with UF 439-16-10-3, a component line of Florunner (Norden et al. 1969). The four-species F_1 complex hybrid was then backcrossed to UF 439-16-10-3 as female, producing a population of BC_1F_1 plants, one of which was designated TP-135-4, and named TxAG-7 (Simpson et al. 1993). Both the lines TxAG-6 and TxAG-7 carry genes for nematode resistance (Nelson et al. 1989; Starr et al. 1990). However, none of the parental genotypes involved in synthesizing the TxAG-6 are ancestors of cultivated groundnut, not the true synthesis of cultivated groundnut. More recently, the amphiploid synthesized by involving progenitor species, *A. duranensis* and *A. ipaënsis*, produced fertile progenies when crossed with *A. hypogaea* (Favero et al. 2006). Work on synthesizing the amphiploids involving wild species, and their subsequent utilization for the genetic amelioration of the cultivated groundnut is in progress at ICRISAT. The synthetic amphidiploids (tetraploid) have been generated from the diploid hybrids involving AB-genome (*A. duranensis* × *A. ipaënsis*, *A. duranensis* × *A. batizocoi*, *A. duranensis* × *A. hoehnei*, *A. valida* × *A. duranensis*, *A.ipaënsis* × *A. duranensis*, *A. batizocoi* × *A. duranensis*, *A. valida* × *A. duranensis*, *A. kempff-mercadoi* × *A. hoehnei*, *A. batizocoi* × *A. cardenasii*, *A. valida* × *A. diogoi*, *A. magna* × *A. batizocoi*, *A. batizocoi* × *A. cardenasii*), AA-genome (*A. kempff-mercadoi* × *A. stenosperma*, *A. duranensis* × *A. cardenasii*) and BB-genome (*A. trinitensis* × *A. hoehnei*, *A. magna* × *A. valida*), which are being further crossed with cultigens to introduce genes of interest into improved genetic backgrounds using marker-assisted introgression to minimize the linkage drag. This "resynthesizing pathway" would allow the breeders to capture the enormous variability available in the wild species by incorporating various exotic genomes in the synthetic amphidiploids, and its subsequent utilization would help in incorporating the traits of interest from various wild species into the cultivated groundnut background [reviewed in Dwivedi et al. (2008)].

Wild *Arachis* species have many undesirable traits linked with resistance traits: thick shell, highly reticulated, constricted, prominently ridged and conspicuously beaked pods, which are small and catenated. Using conventional crossing and selection, it has been difficult to break such undesirable association due to linkage drag while selecting the progenies from such interspecific crosses. However, with the recent developments in marker technology (both in terms of marker developments, SSRs and DArT, and high throughput assay, ABI3700), it should now be possible to minimize the linkage drag, and monitor and fix the allelic variations associated with beneficial traits in progenies from interspecific crosses.

1.10 Outlook

Genetic variability is the key to the success of crop improvement programs. Plant breeders preferably exploit variation from the primary gene pool of a specific crop. Cultivated groundnut has narrow genetic base, probably because of the bottlenecks associated with its origin. Moreover, for some stresses, the resistance is either not available or present in very low levels in *A. hypogaea,* in spite of the fact that over 14,000 germplasm accessions are locked in national and international genebanks. Wild *Arachis,* in contrast, show enormous genetic variation in the traits most important for the enhancement of groundnut productivity. However, most of these variations detected in secondary, tertiary and fourth gene pools require use of techniques such as ploidy manipulation, bridge crosses and ovule/embryo culture. Using these techniques alone or in combinations, researchers have been able to transfer beneficial traits (mostly resistance to pests and diseases) from secondary gene pool to cultivated groundnut. Several elite germplasm from such interspecific crosses that are resistant to pests and diseases have been released worldwide, of which a few have been released as cultivars. Prominent among these are the two root-knot nematode-resistant (carrying gene from wild relatives) groundnut cultivars in the USA. Likewise, some wild *Arachis* species from the tertiary gene pool have been successfully crossed with *A. Hypogaea*; however, the utility of such crosses towards releasing the genetic variation that is useful for selection is yet to be demonstrated. More recently, tetraploid amphiploids, involving several *Arachis* species, including *A. hypogaea* progenitors *A. duranensis* and *A. ipaënsis*, have been produced. These amphiploids are being further crossed with *A. hypogaea* to unlock the genetic variation from *Arachis* species. Over the past few years, molecular biology research in groundnut has made considerable progress towards developing markers (SSR and DArT) and genetic maps. Today, we have large number of SSR markers, the DArT markers being discovered, the high-throughput assay platform (ABI3700), the AA- and BB-genome-based genetic maps involving wild relatives, tetraploid genetic map for *A. hypogaea* (see Sect. 1.6). It is expected that with the availability of these genomic resources, it should be feasible to minimize the linkage drag when selecting progenies with beneficial traits from interspecific crosses. Several projects are underway to exploit these genetic and genomic resources to broaden the genetic base of *A. hypogaea* germplasm for the development of high yielding groundnut cultivars with specific attributes for the benefit of the farming community globally.

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Chapter 2
Cajanus

Nalini Mallikarjuna, K.B. Saxena, and D.R. Jadhav

2.1 Introduction

The cultivation of the pigeonpea goes back to at least 3,000 years. Its center of origin is India (Vavilov 1928; van der Maesen 1980), from where it traveled to East Africa and, by means of the slave trade, to the American continent. Pigeonpea is an ancient crop as there is a mention of pigeonpea in Sanskrit and Buddhist literature dating back to 400 BC to 300 AD (Krishnamurthy 1991). Today, pigeonpea is widely cultivated in all tropical and semi-tropical regions of both the old and the new world.

Pigeonpea is an important grain legume crop of rain-fed agriculture in the semi-arid tropics. The Indian subcontinent, eastern Africa and Central America are the world's three main pigeonpea-producing regions. Pigeonpea is cultivated in more than 25 tropical and subtropical countries, either as a sole crop or as an intercrop with cereals and other legumes. Being a legume, pigeonpea enriches soil through nitrogen fixation. Besides this, it also enriches the soil through the addition of other valuable organic matter and micronutrients. It has a special mechanism to release soil-bound phosphorus from vertisols by secreting pyssidic acid to meet its own as well as that of subsequent crop's phosphorous needs. Pigeonpea has an extensive root system that enables it to tolerate drought and improve soil structure by breaking plow pans. Besides its main use as dry dehulled splits, its tender green seeds and pods are used as vegetable. Its high protein (20–25%) containing leaves are used as fodder and dry crushed seeds as animal feed while the dry stems make quality fuel wood.

Pigeonpea is attacked by a range of biotic (diseases and insect pests) and abiotic (drought, salinity and water logging) factors, which are major constraints to the increased productivity of pigeonpea. Resistance to some of these constraints is not present in the cultivated genotypes, but the wild relatives have been found to be good sources of resistance. Besides this, wild *Cajanus* species have contributed desirable agronomic traits such as cytoplasmic male sterility (CMS) (Mallikarjuna and Saxena 2005; Saxena et al. 2005), dwarf growth habit (Saxena and Sharma 1995) and high protein content (Saxena et al. 2002).

Plant breeding continues to increase the productivity and ensure stable performance of crops in diverse environments. The adoption of genetically homogeneous cultivars has led to diminution of plant genetic diversity. This very process of crop improvement and narrowing of genetic variability is paving the way for epidemics of pests and diseases (genetic vulnerability), as seen in the case of the Phytophthora blight of potatoes in western Europe in 1845–1846 (Gregory 1983), the narrow cytoplasmic base of maize in the USA (Campbell and Madden 1990) and the coffee rust of the 1970s (Damania 2008). Therefore, there is a need of new allelic variation previously not encountered within a crop's domesticated gene pool. Such a situation may arise when attempting to introduce a crop into areas beyond its traditional eco-geographic range, or with the appearance of a new virulent pathogen race, as has been observed in race Ug 99, the stem rust of wheat.

Wild relatives of crop plants are important resources of variability with respect to resistance/tolerance to disease, insect pests and drought, and good agronomic traits; therefore, they could broaden the genetic base of variation of the crop. Whenever

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there is a major epidemic in a region, crop improvement scientists have found resistance to the constraint in the wild relatives of those crops. The recent stem rust of wheat and Phytophthora blight of potato are good examples where scientists have gone back to wild relatives for an integrated approach to tackle the constraints (New Delhi 2008).

It is often said that pigeonpea has reached its performance plateau (Saxena 2008). Although ample morphological diversity is exhibited by pigeonpea as a crop, the same is not true at the molecular level (Yang et al. 2006). The crop has a rich source of variability in the form of wild relatives, which have played a major role in the introduction of disease resistance, good agronomic traits such as high protein content, identification and diversification of cytoplasmic base of CMS system, to name a few.

2.2 Wild Relatives of Pigeonpea

The gene bank at ICRISAT conserves over 13,632 accessions of *Cajanus* species from 74 countries. This includes 555 accessions of wild relatives, which represent six genera and 57 species (Upadhyaya et al. 2007). The majority of the collection has been characterized for morpho-agronomic traits of importance in crop improvement.

Pigeonpea, *Cajanus cajan* L. belongs to the subtribe *Cajaninae*, which contains 13 genera. Earlier, the genus *Atylosia* and *Cajanus* were considered closely related, however, recently the genus *Atylosia* has been merged with the genus *Cajanus* (van der Maesen 1980). Subsequently, the genus *Cajanus* has 32 species, 18 of which are endemic to Asia and 13 to Australia and one to western Africa (van der Maesen 1986). Apart from these, there are other related genera, namely *Rhynchosia*, *Dunbaria*, *Flemingia*, *Paracalyx*, *Eriosema*, *Adenodolichos*, *Bolusafra*, *Carissoa*, *Chrysoscias* and *Baukea*. Figure 2.1 depicts the relationships among the wild species according to their crossability with cultivated species. *Cajanus* species, which are endemic to Australia, are *Cajanus lanceolatus*, *C. confertiflorus*, *C. viscidus*, *C. acutifolius*, *C. aromaticus*, *C. crassicaulis*, *C. lanuginosus*, *C. latisepalus*, *C. reticulates*, *C. pubescens*, *C. cinereus*, *C. marmoratus* and *C. mareebensis*, and *C. kerstingii* is endemic to Africa.

2.2.1 *Gene Pools of* Cajanus

Harlan and de Wet (1971) proposed a systematic means of grouping the germplasm of a crop species

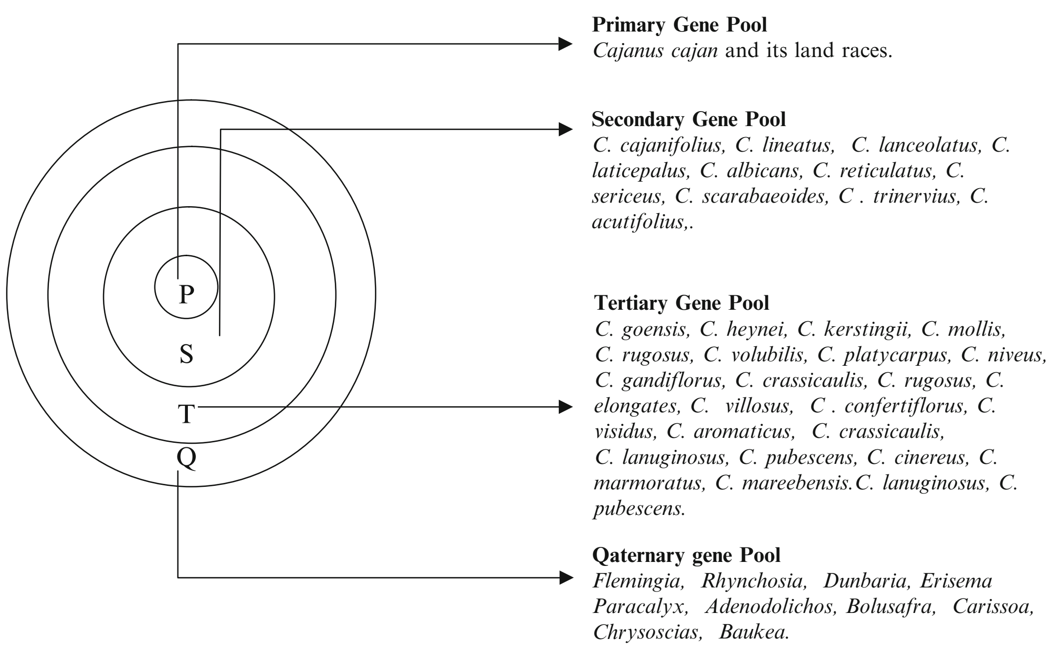


Fig. 2.1 Gene pools of the genus *Cajanus*

and their wild relatives. They constituted three basic gene pools and divided them as primary gene pool (GP1), secondary gene pool (GP2) and tertiary gene pool (GP3) and the quaternary gene pool (GP4).

2.2.2 Primary Gene Pool

The primary gene pool consists of cultivated species and its landraces. The germplasm in the primary gene pool are fairly easy to use; however, a perusal of the utilization pattern of *Cajanus* germplasm indicates that a very small proportion of germplasm has been used so far in pigeonpea improvement programs, globally. In pigeonpea, 57 ancestors were used to develop 47 varieties. The top ten ancestors contributed 48% to the genetic base of the released varieties (Kumar et al. 2003). One of the reasons for such poor utilization may be that in spite of the vast number of lines available in the primary gene pool, there is a lack of characterization, evaluation and genetic diversity data.

As the accessibility and utilization of a collection is inversely related to its size (Frankel and Soule 1981), a core collection of pigeonpea, which represents the genetic spectrum that is representative of >85% of the diversity of the entire collection, was developed (Reddy et al. 2005). This core collection has been characterized for phenotypic traits (Upadhyaya et al. 2007). The information generated in the development of core collection has shown that it is possible to further reduce the size of the collection into a mini core, which would have 1% of the collection. This will provide options to breeders to use the germplasm as parents, which will enhance the trait(s), besides broadening the genetic base of variation in the cultivars without hindering the progress of breeding programs.

To capture maximum diversity, a composite collection of *Cajanus* that consists of 1,000 accessions has also been developed through a well-directed Generation Challenge Program. This composite collection consists of a few accessions from wild species, the core collection and accessions with traits of economic importance and resistance to major biotic and abiotic stresses. This composite collection will be genotyped using 20 polymorphic simple sequence repeat (SSR) markers to know the structure of the population. The genotyping data will be used to select a 300-accession reference collection for use by the global scientific community (*http://www.generationcp.org/sccv10/sccv10_upload/2005_annual_report.pdf*).

Many pigeonpea cultivars have shown important characters such as resistance to Alternaria blight, wilt, sterility mosaic disease (SMD) and Phytophthora blight (Sharma et al. 1987). Germplasm lines from different parts of India have contributed dwarfing genes with a recessive mode of gene action (Saxena et al. 1989). ICP 7035, a popular vegetable-type pigeonpea, with high sugar content and SMD resistance, is a line collected from Madhya Pradesh, India.

2.2.3 Secondary Gene Pool

The greatest contribution to the utilization of wild species for pigeonpea improvement is from this group as the species are cross-compatible, which means there would be chromosome recombination and transfer of useful traits/genes from wild *Cajanus* species. There are ten wild species in the secondary gene pool (Fig. 2.1), and each wild species has several collections. The accessions of a species are important sources of genetic diversity with the presence of useful traits (Saxena et al. 1996; Upadhyaya 2006; Sujana et al. 2008). The introgression of useful genes/traits from secondary gene pool species is carried out through conventional hybridization techniques. In general, the techniques such as hormone-aided pollinations and embryo rescue are not essential, but sometimes these techniques are necessary to obtain more hybrid seeds, as was done in the case of a cross involving *C. acutifolius* and *C. cajan* (Mallikarjuna and Saxena 2002). A number of wild species of this group have been used in the genetic improvement of pigeonpea, including development of unique cytoplasmic nuclear male sterile systems (CMS), high protein lines, dwarf plant stature, disease and pest-resistant lines.

2.2.3.1 Cytoplasmic Nuclear Male Sterile Systems

Five unique CMS systems have been developed for pigeonpea. These are A_1 cytoplasm derived from *C. sericeus* (Ariyanayagam et al. 1995). The CMS lines derived from this source are sensitive to temperature

changes. The male sterile plants change to male fertile under low-temperature conditions (Saxena 2005). Although the A_1 source produces good yield, the presence of fertile plants in the progeny prevents it from becoming a desirable source for the development of CMS system. The A_2 cytoplasm derived from *C. scarabaeoides* (Tikka et al. 1997; Saxena and Kumar 2003) is a stable source of CMS. The drawback of this system is that fertility restorers are inconsistent across environments. Hybrids derived from A_2 showed high heterosis for yield (IIPR 2007). Unstable seed set across environments is an undesirable character of this source. A_3 cytoplasm derived from *C. volubilis* (Wanjari et al. 2001) does not have quality fertility restoration system. Hence, this source is not popular as a cytoplasm to develop CMS system. The A_4 cytoplasm was derived using *C. cajanifolius* (Saxena et al. 2005). The system is stable across environments with very good fertility restoration system. The A_4 system is used at ICRISAT and by other pigeonpea breeders of India to exploit heterosis in pigeonpea. Crosses between *C. cajan* and *C. acutifolius* gave rise to CMS on cultivated pigeonpea cytoplasm, which was named as A_5 (Mallikarjuna and Saxena 2005). It is fully maintained by its male parent *C. acutifolius,* and most of the cultivated types restore fertility. The A_5 cytoplasm is still under development. Recently, crosses between *C. platycarpus* and cultivated pigeonpea gave rise to open flower (cleistogamous) segregants (Mallikarjuna et al. 2006). Some of the progeny were completely male sterile with white anthers. In the semi-fertile progeny, pollen shedding was not observed as the anthers had a thick cell wall. Self-pollination did not set seeds but seed set was observed when pollinated with a range of other cultivars. This may be another source of CMS in pigeonpea (Mallikarjuna unpublished results).

2.2.3.2 Cleistogamy

Pigeonpea is partially out crossing and insects mediate the process. The process of out-crossing is important in the development of CMS systems in pigeonpea but can lead to genetic deterioration. A partially cleistogamous line, which showed less than 1% cross-pollination, was purified from the cross *C. cajan* × *C. lineatus*, which was governed by a single recessive gene (Saxena et al. 1992). Partial cleistogamous lines developed from the above cross were found to be stable in India as well as in Sri Lanka. Cleistogamous trait can be utilized in pigeonpea to obtain pure seeds from genetic stocks.

2.2.3.3 High Protein and Seed Weight

High protein line, ICPL 87162, was developed from the cross *C. cajan* × *C. scarabaeoides* (Reddy et al. 1997). Dhal protein content of ICPL 87162 ranged from 30 to 34% compared to 23% in the control cultivar. ICPL 87162 is resistant to sterility mosaic disease but is susceptible to wilt. High protein breeding lines were developed from *C. sericeus*, *C. albicans* and *C. scarabaeoides*. Significant positive correlation between seed size and protein content was observed in lines derived from *C. scarabaeoides*. Lines HPL 2, HPL 7, HPL 40 and HPL 51 were some of the high protein and high seed weight lines derived from wild species (Saxena et al. 1987). More recently, crosses between pigeonpea and *C. acutifolius* yielded progeny with high seed weight. High seed weight accompanied by beige seed color is a desirable trait. The material is under multilocational testing (Mallikarjuna unpublished results).

2.2.3.4 *Helicoverpa armigera* Resistance

Cajanus scarabaeoides, *C. acutifolious*, *C. sericeus* and *C. albicans* are some of the wild *Cajanus* species with resistance to pigeonpea pod borer *H. armigera* (Sujana et al. 2008). *C. scarabaeoides*, a wild species of Indian origin, has multiple disease resistance (Kulkarni et al. 2003; Upadhyaya 2006). Pods of *C. scarabaeoides* have a dense covering of non-glandular and low density of glandular trichomes (Shanower et al. 1997). Since *C. scarabaeoides* had least damage compared to cultivated pigeonpea, it was concluded that non-glandular trichomes form a preventive layer for insect lodging and feeding on the pod surface. Further research is necessary to know the differences between different glandular and non-glandular trichomes to assign clear-cut influences of these trichomes. As large number of glandular trichomes are present on *C. cajan* pods, they may be playing a role in the high damage due to pod borers. *C. scarabaeoides* was used as a wild species to introgress resistance to sterility mosaic disease (Patancheru isolate) and

H. armigera insect (Mallikarjuna unpublished results). *C. acutifolius*, a wild species native of Australia, can be crossed with pigeonpea as a one-way cross. The reciprocal cross, using *C. acutifolius* as the female parent, aborts to give rise to immature seeds. In vitro interventions are necessary to obtain hybrid plants (Mallikarjuna and Saxena 2002). Advanced generation population from cross utilizing *C. acutifolius* as the pollen parent has shown resistance to pod borer damage (Mallikarjuna et al. 2007), variation in seed color and high seed weight (Fig. 2.2). Some lines have shown high level of resistance to pod borers, pod fly and bruchids under unprotected field conditions (Table 2.2).

Some of the other important traits identified in wild *Cajanus* are nematode resistance, Alternaria blight resistance (Sharma et al. 1987) and salinity tolerance (Subbarao 1988; Srivastava et al. 2006).

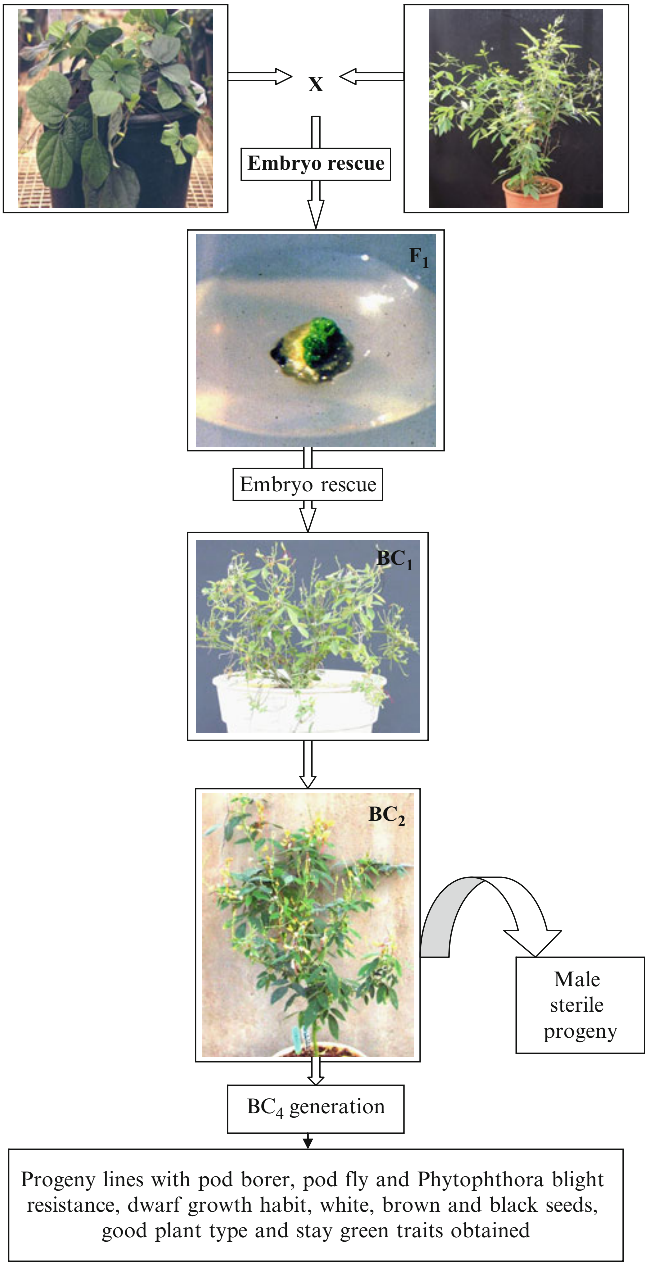


Fig. 2.2 Tapping useful genetic variation from *Cajanus platycarpus*

2.2.3.5 Sterility Mosaic Disease Resistance

Sterility mosaic disease (SMD) of pigeonpea is transmitted by eriophyid mites, *Aceria cajani*, the smallest arthropods, transmitting the virus called tenui-like virus (PPSMV). Infected pigeonpea plants show mosaic symptoms on the leaves and cease flowering, rendering the plants sterile with no pod formation. Until recently, the causal agent of SMD was not identified, but it was possible to identify resistant plants as well as segregating populations based on disease symptoms. There are three major isolates of this virus and amongst these the Bangalore isolate has been identified as the most virulent virus and sources of resistance are few. Lava Kumar et al. (2005) have shown that many of the wild *Cajanus* species are resistant to all the isolates of the SMD virus, and this resistance to SMD is monogenic and recessive (Kulkarni 2002). *C. scarabaeoides* (ICPW 94), which is resistant to all the isolates of SMD, was used in the crossing program, and the progeny were tested for resistance. Many of the plants were found to be disease-free and were classified as resistant. Some of the plants showed relatively mild disease symptoms, called as ring spots, and these were classified as moderately resistant. These plants flowered and set seeds. The susceptible plants had disease mosaic symptoms with crinkled leaves and did not flower and set seeds (Mallikarjuna and Wesley unpublished results). Lines derived from crosses with *C. acutifolius* and *C. platycarpus* have shown resistance to Patancheru isolate of SMD under field conditions (Saxena and Mallikarjuna unpublished results).

2.2.4 Tertiary Gene Pool

There are 20 wild species in the tertiary gene pool of pigeonpea (Fig. 2.1). Till date, only one wild *Cajanus* species from this gene pool is amenable to interspecific hybridization and gene transfer (Mallikarjuna and Moss 1995; Mallikarjuna et al. 2006). An important prerequisite for successful cross-pollinations using incompatible species is the application of growth regulators to pollinated pistils (Mallikarjuna 2003) followed by embryo rescue of aborting hybrid embryos (Mallikarjuna 1998). Embryo rescue technique is used to save aborting hybrid embryos in vitro. The immature aborting embryo is removed from seeds and cultured in vitro to produce hybrid plants. Hormone-aided pollinations and embryo culture have been valuable tools for the transfer of Phytophthora blight resistance from *C. platycarpus*, a wild species from the tertiary gene pool of pigeonpea, into pigeonpea (Mallikarjuna et al. 2006).

Wide crosses with distantly related species give rise to novel variation, not observed in either of the parents used in the crossing program (Hoisington et al. 1999). In the BC_2 plants, the flower color varied from yellow to orange-colored petals. Pollen fertility varied from 27 to 46% (Table 2.1). Some plants had open flowers, unlike those observed in pigeonpea or *C. platycarpus* (Cherian et al. 2006). Open flowers of pigeonpea is likely to play an important role in the development of hybrid pigeonpea as this trait will facilitate cross-pollination. Seed color ranged from white to black.

A selection was made in the BC_2 generation for open flower morphology and low pollen fertility, and this line was called F_1BC_2-E (Fig. 2.3). They were backcrossed with the recurrent parent pigeonpea cv. ICPL 85010. Two lines were observed to have total pollen sterility. Their progeny were also completely male sterile. Seeds from self-pollinations were not obtained, and forced self-pollinations did not set seeds. The flowers had white anthers with open flower morphology (Fig. 2.3: E15 and E4). Anthers had shrunken pollen sacs with no pollen. Some of the anther sacs had some pollen (Fig. 2.3: E15), but the anthers never dehisced to release the pollen grains.

Table 2.1 Analysis of morphological traits in progeny lines derived from *C. platycarpus*

Identity	Plant habit	Flower color	Flower morphology	Seed color	Pollen fertility
F1BC2-A	Erect	Orange keel	Closed	Brown	46
F1BC2-B	Semi-erect	Orange keel	Closed	Brown	30
F1BC2-C	Erect	Orange keel	Closed	Brown	33
F1BC2-D	Erect	Red keel	Open	Brown	33
F1BC2-E	Erect	Red keel	Open	Black	27

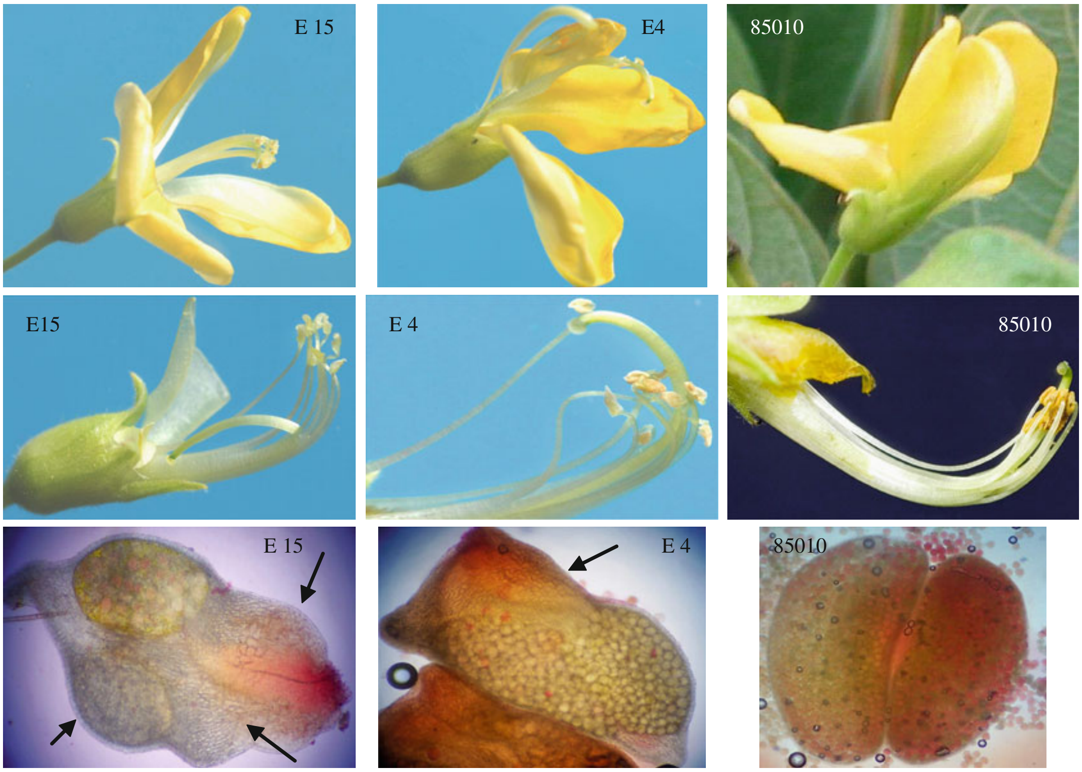


Fig. 2.3 Male sterility in the progeny from the cross *C. platycarpus* × *C. cajan*. E15: Flower, anther bundle and single anther. E 4: Flower, anther bundle and anther. 85010: Flower, anther bundle and single anther

Male sterility coupled with open flowers are traits important for the development of CMS systems as open flowers would favor cross-pollinations. None of the CMS systems available for pigeonpea have open flowers. Experiments are underway to identify restorers of fertility and maintainers of male sterility.

The progeny lines derived from F_1BC_2-A was back-crossed to cultivated recurrent parent ICPL 85010 and F_1BC_4-A lines were developed. Progeny lines were screened for days to flower, which varied from 60 to 92 days. In the parental lines, *C. platycarpus* flowered at 50 days and the cultivar ICPL 85010 flowered at 83 days. There was improvement in 100-seed weight compared to *C. platycarpus* (6.1 g/100 seeds). Three lines F_1BC4-A10-7, F_1BC4-A17-8 and F_1BC4-A14-6 had higher 100-seed weight than both the parents (Table 2.2). These might be good sources of bold seeds in pigeonpea. Protein content in all the hybrid lines was more than that in *C. platycarpus*, and F_1BC_4-A4 and F_1BC_4-A19-14 showed marginally more than that in the cultivated parent. Some of the lines (F_1BC_4-A8-4 and F_1BC4-A14-6) had a tendency towards male sterility with pollen fertility not exceeding 30% with open flowers and non-dehiscent anthers. Non-dehiscent anthers in open flowers coupled with high pollen sterility are desirable traits of a CMS source.

All the lines were screened for *H. armigera* (pod borer), *Melanagromyza obtusa* (pod fly) and *Callosobruchus chinensis* (bruchids) under unprotected field conditions. Damage due to *H. armigera*, in the wild parent *C. platycarpus*, was less than 1%. Damage in cultivated parent ICPL 87 was 69%. Damage in F_1BC_4-A derivatives ranged from 2 to 37% with majority of the lines with less than 15% damage (Table 2.2). It was observed that there were significant differences between the lines for pod borer and bruchid resistance and 100-seed weight (Table 2.2). The results show that there is good scope to transfer *H. armigera* resistance from *C. platycarpus*. Line F_1BC_4-A19-14 has pod borer and bruchid resistance (Table 2.2), and marginally high protein was an additional desirable trait present in the line. Line F_2BC_4-A22 plants consistently showed short stature

Table 2.2 *Cajanus platycarpus* progeny showing insect resistance and seed weight

Line no.	Yield components				Biotic stresses					
	Healthy pods pl^{-1} (no.)		100-seed wt (g)		Pod borer damage (%)		Pod fly damage (%)		Bruchid damage (%)	
F1BC4-A4 10-7-1	81.3 ± 35.81	gh	10.29 ± 1.15	bc	9.91 + 7.11	ijklm	14.54	defgh	1.03	jkl
F1BC4A4 10-12-1	99.5 ± 90.42	cde	9.82 ± 0.76	ef	16.61 + 7.77	ef	12.05	hij	2.12	fghj
F1BC4A4 13-2-1	91.25 ± 27.35	g	9.44 ± 1.40	ghi	11.12 + 9.09	ghijklm	15.84	de	2.74	fgh
F1BC4A4 13-2-1	63.2 ± 26.19	hij	8.64 ± 0.61	l	10.14 + 7.55	ijklm	10.24	ijk	0.04	l
F1BC4A4 13-5-1	79.27 ± 31.12	h	9.52 ± 0.85	ghi	12.59 + 6.81	fghijkl	12.85	fghi	6.28	cde
F1BC4A4 13-5-1	70.55 ± 27.29	hi	9.11 ± 0.73	jk	6.85 + 4.45	m	12.52	ghi	0.23	kl
F1BC4A4 14-16-1	95.94 ± 63.37	cdef	9.22 ± 0.92	ij	14.67	efghi	14.52	efgh	1.55	hjk
F1BC4A4 14-21-1	74.33 ± 47.75	hi	8.56 ± 6.59	l	10.26	ijklm	7.68	k	7.44	bcd
F1BC4A4 14-18-1	118.22 ± 76.41	a	10.27 ± 0.72	bcd	9.71	jklm	12.94	fghi	1.38	hjkl
F1BC4A4 14-4-1	72.05 ± 41.13	hi	9.70 ± 0.82	efg	18.56	cde	9.48	jk	1.98	ghj
F1BC4A4 14-6-1	106.93 ± 84.15	abc	9.92 ± 0.96	e	15.89 + 7.71	b	10.80	ij	1.01	jkl
F1BC4A4 14-6-1	54.50 ± 30.17	jk	9.85 ± 0.82	ef	24.12 + 15.07	efg	10.64	ijk	0.47	kl
F1BC4A4 14-9-1	111.35 ± 79.42	ab	8.64 ± 0.87	l	13.18	fghijkl	14.96	defg	3.50	f
F1BC4A4 15-14-1	73.52 ± 35.28	hi	9.60 ± 0.88	fghi	13.42	fghijk	3.73	l	2.01	ghj
F1BC4A4 17-1-1	50.15 ± 25.25	jk	8.82 ± 0.69	kl	9.43	jklm	16.68	de	0.13	kl
F1BC4A4 17-5-1	67.6 ± 39.04	hi	9.14 ± 0.73	ij	13.28	fghijkl	14.61	defgh	0.00	l
F1BC4A4 17-8-1	73.11 ± 41.36	hi	11.02 ± 1.62	a	11.42	ghijklm	10.98	ij	14.33	a
F1BC4A4 19-1-1	76.00 ± 49.35	h	9.61 ± 0.89	fghi	9.46	jklm	7.74	k	7.69	bc
F1BC4A4 19-12-1	77.95 ± 36.69	h	9.42 ± 1.10	ghi	7.23	m	15.71	def	8.65	b
F1BC4A4 19-14-1	8.54 ± 7.29	m	9.36 ± 0.43	ij	15.25	efgh	41.75	a	0.00	l
F1BC4A4 19-20-1	22.82 ± 7.59	cdef	10.46 ± 0.99	b	22.85	bc	16.57	de	2.52	fgh
F1BC4A4 19-8-1	99.7 ± 71.36	cd	9.28 ± 1.08	ij	14.18	efghij	11.48	ij	1.06	jkl
F1BC4A4 20-10-1	34.17 ± 24.76	l	9.98 ± 1.66	de	21.52	bcd	21.65	b	0.33	kl
F1BC4A4 20-5-1	10.54 ± 7.42	hi	9.82 ± 0.63	ef	10.55	ijklm	20.19	bc	0.00	l
ICPL 85010 (S)	12.00 ± 0.93	m	7.66 ± 0.93	m	68.00	a	17.41	cd	3.10	fg
Mean ± SE	74.26 ± 5.48		9.48 ± 0.14		15.61 ± 2.36		14.30 ± 1.39		2.78 ± 0.70	
CD (0.05)	11.31		0.29		4.88		2.87		1.45	

Means within the same row with same letter are not significantly different ($P < 0.05$)

with bushy growth habit, a trait not observed in the rest of the progeny.

Screening thousands of germplasm lines for Phytophthora blight, especially for race P_3, has failed to identify lines with resistance. Race P_3 is the most virulent race. Screening wild *Cajanus* for Phytophthora blight disease has resulted in the identification of *C. platycarpus*, which has shown resistance to all isolates of Phytophthora blight fungi. Although *C. platycarpus* belongs to the tertiary gene pool of pigeonpea, it has been successfully crossed, and progeny have been generated at ICRISAT (Mallikarjuna et al. 2006). Screening interspecific derivatives to Phytophthora blight disease under glasshouse conditions has shown that it is possible to transfer resistance from *C. platycarpus* (Mallikarjuna et al. 2005). Tetraploid progeny from F_1 hybrid *C. platycarpus* × *C. cajan* showed high level of resistance to Phytophthora blight disease, under both field and glasshouse-simulated conditions. As it was not possible to backcross them to pigeonpea, the progeny is best suited as a ground cover due to its semi-trailing growth habit. These results show that there is ample scope to transfer resistance from wild *Cajanus* into the cultivated *Cajanus* species.

It is hoped that the techniques developed for the cross *C. platycarpus* × *C. cajan* will be useful to cross other wild *Cajanus* species from the tertiary gene pool with cultivated *C. cajan*.

2.2.5 Quaternary Gene Pool

Wild species placed in the quaternary gene pool of *Cajanus* belong to different genera, such as *Flemingia*, *Rhynchosia*, *Dunbaria* and *Eriosema*, to name a few (Fig. 2.1). Results of an exhaustive crossing experiment have shown that some of the species in

this group may be amenable to hybridization with pigeonpea; however, these results need to be confirmed (Mallikarjuna unpublizhed results). Until more cross-ability studies are carried out using species from this gene pool, it may not be possible to access genes/traits from this gene pool for pigeonpea improvement. Isolation of genes from wild species, especially from the quaternary gene pool, may be an important strategy to introduce genes through genetic transformation, which are not amenable to wide crosses research. Alternatively, protoplast fusion may be an important technique to introduce genes/traits from this gene pool.

2.3 Genetic Diversity in the Genus *Cajanus*

Biochemical markers have been effectively used to detect polymorphism. Krishna and Reddy (1982) used esterase isozymes to study species affinity between pigeonpea and a few of the wild relatives. Esterase isozymes studies showed affinity between wild species *C. scarabaeoides*, *C. albicans*, *C. scarabaeoides*, *C. sericeus* and *C. volubilis* with closer affinity between *C. albican* and *C. scarabaeoides*. *C. platycarpus* had distinct band and did not show affinity with any of the wild species used in the study or with pigeonpea. *C. cajanifolius* showed a closer affinity to *C. cajan*. Panigrahi et al. (2007) carried sodium dedocyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of seed albumins and globulins of 11 *Cajanus* species including cultivated species *C. cajan*. Banding patterns revealed *C. cajanifolius* to be the closest to *C. cajan*, with *C. platycarpus* as an outgroup species justifying its status as a tertiary gene pool species (van der Maesen 1986). The study also showed *C. cajan* sharing homology with *C. cajanifolius* and also with *C. scarabaeoides*, *C. albicans*, *C. volubilis* and *C. sericeus*. These results indicate that pigeonpea is a product of multigenomic interaction involving *C. cajanifolius*, *C. scarabaeoides* and other species.

Boehringer et al. (1991) used allozymes and were able to detect polymorphism between Indian and Zambian genotypes of pigeonpea. Nadimpalli et al. (1994) used restriction fragment length polymorphism (RFLP) markers to determine phylogenetic relationships among 12 species belonging to four related genera. Two closely related *Cajanus* species, *C. scarabaeoides* and *C. cajanifolius*, showed a close relationship with each other; amongst the two, *C. cajanifolius* was closer to *C. cajan*. Interestingly, species belonging to different genera grouped together and were away from the above group. Species belonging to *C. lineatus*, *C. albicans* and *C. sericeus* formed a group that had a closer relationship with the first group. Utilizing random amplified polymorphic DNA (RAPD) markers, it was possible to distinguish pigeonpea cultivars, albeit with low levels of polymorphism (Ratnaparkhe et al. 1995). High level of polymorphism was observed between different species of *Cajanus* with *C. albicans*, *C. sericeus* and *C. lineatus*, which are of Indian origin, showing closer relationship to *C. cajan* than to *C. acutifolius*, *C. grandifolius* and *C. reticulates*, which are of Australian origin. *Rhynchosia* species grouped together, with *Flemingia stricta* being distinct from rest of the species used in the study. Punguluri et al. (2006) used amplified fragment length polymorphism (AFLP) markers to study genetic diversity in pigeonpea cultivars that were found to have low level of diversity (87% common bands), but they were able to distinguish Pusa cultivars from others. Genetic distance between wild relatives *C. volubilis* and *Rhynchosia bracteata* was high, and also from the cultivated pigeonpea. Ribosomal genes from wheat and *Vicia faba* were used to distinguish pigeonpea cultures and some wild relatives. The probes were not able to distinguish cultivars, but polymorphism was observed between species but not within species. The study showed a close relationship between *C. cajan* and *C. scarabaeoides*, and they, in turn, were related to *C. mollis* and *C. albicans*. *C. reticulates* showed 95% similarity with *C. platycarpus*. This study concluded that *C. Scarabaeoides* is closer to *C. cajan* than *C. cajanifolius* (Parani et al. 2000). In conclusion, genetic diversity studies show that two wild relatives, *C. cajanifolius* and *C. scarabaeoides*, are closely related to pigeonpea than any of the compatible wild species of the genus.

The merger of genus *Cajanus* with *Atylosia* has strong cytological support with the same chromosome number in all the species being $2n = 22$ (Deodikar and Thakar 1956; Dundas 1990). Chromosome number analysis of 20 species belonging to five genera namely *Cajanus*, *Rhynchosia*, *Dunbaria*, *Flemingia* and *Paracalyx* showed $2n = 22$ chromosome number (Ohri and Singh 2002).

There is further evidence from cytology that *C. cajanifolius* is the progenitor species of *C. cajan* as the two have similar karyotype, and the hybrids between the two species show normal meiosis with high pollen fertility and high seed set (Pundir and Singh 1985). Hybrids between *C. cajan* and wild species *C. scarabaeoides*, *C. albicans*, *C. sericeus* and *C. acutifolius* showed 0–2 univalents with mature seed set (Pundir and Singh 1985). The presence of univalents shows that the genomes of *C. cajan* and the above-mentioned wild *Cajanus* species are more divergent than *C. cajanifolius*. The reciprocal crosses involving *C. lineatus* (Mallikarjuna unpublished results) and *C. acutifolius* did not set mature seeds. The aborting F_1 embryos from the cross *C. acutifolius* × *C. cajan* were germinated in vitro and hybrid plants obtained. In spite of normal chromosome segregation at metaphase in 96% of the meiocytes, pollen fertility was only 12–16% (Mallikarjuna and Saxena 2002).

Analysis of the F_1 hybrid between *C. platycarpus* and *C. cajan* showed a mean of six univalents and eight bivalents. The presence of six univalents shows that the genomes of *C. platycarpus* and *C. cajan* are divergent with 2–3 non-pairing chromosomes. Pollen fertility in the hybrid was 0.05%, which again shows that the two genomes are not closely related (Mallikarjuna et al. 2006). The placement of *C. platycarpus* in the tertiary gene pool of pigeonpea is therefore justified.

2.4 Genomic Resources

Molecular markers are an important resource to study the geographical origin, genotype identification and genetic diversity, molecular linkage map, gene synteny, trait tagging and marker-assisted selection, association mapping, map-based cloning. RAPD technique was used to identify parents from hybrids of the cross *C. platycarpus* × *C. cajan* (Mallikarjuna 2003). Although RAPDs are not favored as compared to other markers, they can still be effectively used to distinguish parents and hybrids. Kotresh et al. (2006) used RAPDs to show association between markers and Fusarium wilt resistance. Until now, there were only ten SSR markers, which could be used to detect variation in pigeonpea (Burns et al. 2001). In the study by Odeny et al. (2007), 208 SSR loci were identified by screening a non-enriched partial genomic library. Primers were designed for 39 SSR loci, 20 of which amplified PCR products of the expected size. Nineteen of the primer pairs were polymorphic amongst 15 cultivated and nine wild *Cajanus* accessions. A community effort was undertaken (Dubey et al. 2009) to develop more SSR markers. Several SSR-enriched genomic DNA, cDNA and bacterial artificial chromosome (BAC) libraries were developed from leading varieties of pigeonpea. A total of 86,268 BAC-end sequences were generated that provided 9,956 pseudo-contigs and 42,285 singletons. A large number of SSR markers are being developed from BAC-end sequences and SSR-enriched libraries. By using 454/FLX sequencing on the normalized cDNA pool from 20 tissues representing different developmental stages, a total of 496,705 sequence reads have been generated to provide approximately 22,000 unigenes. Once SSR markers are developed from this study, the crop will be on par with other legumes such as chickpea, which has more than 400 SSR markers (Lichtenzveig et al. 2005).

Diversity array technology (DArT) is a novel genome-wide genotyping method. It offers low-cost, high-throughput and sequence-independent genotyping. Yang et al. (2006) reported the development and application of DArT for pigeonpea. DArT analysis showed no clear differentiation among cultivars from different regions, with cultivars from Africa showing some diversity. There was differentiation between wild and cultivated species. They inferred that morphological variation observed in cultivated pigeonpea accessions was much higher than that observed at the molecular level, whereas the wild species of pigeonpea and its related genera exhibited a higher degree of molecular diversity than that observed at the morphological level.

A beginning has been made to develop advanced backcross QTL (AB-QTL) analysis as proposed by Tanksley and Nelson (1996). In this approach, a wild species is crossed with the elite cultivar and backcrossed once or twice (sometimes more) with the elite cultivar, and selfed for one or two generations (sometimes more). The segregating BC_1F_2/BC_2F_2/BC_2F_3 lines are phenotyped for traits of interest and genotyped with polymorphic markers. This is a method for transferring agronomically important quantitative traits from wild species to the cultivated species. The approach has great potential to harness the wealth of wild relatives for pigeonpea improvement, where the cultivated species show low level of

polymorphism and susceptible to major diseases and insect pests.

2.5 Conclusion

Pigeonpea is an important protein rich food of vegetarian diet. It is a favorite crop of small holder farmers as the crop can tolerate and yield high under drought conditions when many other crops fail. Pigeonpea yield has reached a plateau and is susceptible to a range of diseases caused by virus, fungi and bacteria. Although high degree of morphological variability is seen, the same is not true at the molecular level. Crop improvement programs are looking for increased genetic diversity by tapping wild relatives from different gene pools. There is enough evidence to prove that *C. cajanifolius* is the progenitor species of pigeonpea. The secondary gene pool has contributed various traits for the improvement of the crop. In spite of the success obtained in the utilization of wild relatives from the secondary gene pool, there is scope to use others, which has not been attempted in the crossing program. Progress has been made to exploit and introgress useful traits including male sterility from *C. platycarpus*, a tertiary gene pool wild relative of pigeonpea. This has opened up avenues to tap other species in the tertiary gene pool. There are many species in the tertiary gene pool of the genus *Cajanus*. Many of them have not yet been crossed with pigeonpea. It is possible that some of the species placed in the tertiary gene pool may move to secondary gene pool, if they are cross-compatible with cultivated pigeonpea. Enhanced genomic resources may be available in the near future as there is international collaboration to develop them.

2.6 Future Prospects

Pigeonpea is a source of protein for vegetarian diet and resource poor farmers in the rainfed tropics. It has built in resilience to withstand drought and can yield even under very low input conditions. Efforts to broaden the genetic base and introduce traits for various biotic stresses and desirable abiotic traits have been significant. There is renewed interest to exploit more wild relatives from the secondary gene pool, and such efforts would have a big impact on broadening the genetic base of variation of pigeonpea and introduction of useful biotic, abiotic and agronomic traits. The possibility of exploiting wild relatives from the tertiary gene pool has opened up new vistas for the broadening of the genetic base of variation and for improvement in pigeonpea. Development of genomic resources has gained new impetus with community effort, and the development of genome-wide markers may open avenues for molecular marker-assisted gene introgressions and breeding.

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Chapter 3
Chenopodium

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3.1 Introduction

Chenopodium, commonly known as the goosefoot genus, includes a wide array of species and is native to all the inhabited continents as well as far-flung archipelagoes like Juan Fernandez, New Zealand, and Hawaii (Table 3.1). Most of these species are facultatively autogamous annuals, having a base chromosome number of $x = 9$. Many *Chenopodium* species are adapted to arid and/or saline environments. The genus is notorious for its invasive weeds, such as lambsquarters or pigweed (*C. album* and *C. berlandieri*), although these and at least two other species of the genus were domesticated anciently on four continents, as both vegetable and seed crops, while numerous species provided vegetative sustenance for hunter-gatherers from antiquity, particularly in arid regions like the North American west and northern Australia (McConnell 1998). One of these, South American quinoa (*C. quinoa*), has risen from a neglected subsistence crop of indigenous farmers to become a major export of the Andean nations of Bolivia and Peru within the past 20 years. The emergence of quinoa to prominence in organic food markets of the Developed World has led to scientists giving increasing attention to the crop's unique nutritional benefits, including a superb amino acid balance in the seed, and potentially novel abiotic stress-tolerance mechanisms. There is also growing curiosity regarding four lesser known domesticated *Chenopodium* crops: South American *cañahua* (*C. pallidicaule* Aellen), Mexican *huazontle* and *chia roja* [*C. berlandieri nuttaliae* (Safford) H.D. Wilson and Heiser], and Himalayan *khan* or *bithua* (*C. album* L. or *C. giganteum* D. Don; Partap et al. 1998). Quinoa geneticists recognize the tremendous value of this plethora of exotic germplasm for improving the South American crop and for expanding quinoa production into new environments. The novel biochemical and physiological characteristics in quinoa should be expressed at even greater levels in other *Chenopodium* species, making them attractive as natural product sources to biochemists and as unique gene resources to crop breeders.

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3.2 *Chenopodium* Taxonomy

Aellen and Just (1943) published a detailed classification system of American chenopods. Aellen (1960) later expanded this system to encompass 120 worldwide species. The hierarchy of divisions and subdivisions for this taxonomy (Table 3.2) provided the basis for most of the *Chenopodium* systematic work until the late 1990s, when a new system was devised by Mosyakin and Clemants (1996, 2002, 2008); Table 3.3. The primary alteration of the latter system was the removal from the genus of Sections *Ambrina*, *Botryoides*, *Orthosporum*, and *Roubieva* into a separate genus, *Dysphania*. We will defer to this latter taxonomic system for all discussion related to North American taxa as per the Online Flora of North America (Clemants and Mosyakin 2003; *http://www.efloras.org*).

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Table 3.1 Known *Chenopodium* species and their putative origins

Species	Common name	Habit	Putative origin	References
acuminatum Willd.		Wild	Eurasia	Flora of China in http://www.efloras.org
albescens Small		Wild	N. America	Clemants and Mosyakin (2003)
***album* L.**	Lambsquarters, fat hen	Weed, domesticate	Eurasia	Clemants and Mosyakin (2003), Reiche in http://www.efloras.org, Flora of China in http://www.efloras.org, Flora of Pakistan in http://www.efloras.org, Mulgura (1999) and Marticorena (2010)
atripliciforme Murr		Wild	Eurasia	Flora of Pakistan in http://www.efloras.org
***atrovirens* Rydb.**	Pinyon goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
auricomiforme Murr and Thell.		Wild	Australia	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au
auricomum Lindl.	Queensland bluebush	Wild (perennial)	Australia	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au
badachschanicum Tzvelev		Wild	Eurasia	Flora of Pakistan in http://www.efloras.org
***berlandieri* Moq.**	Pitseed goosefoot	Weed, wild	N. America	Clemants and Mosyakin (2003)
***berlandieri* ssp. *nuttaliae* (Safford) H.D. Wilson and Heiser**	Huazontle, chia roja, quelite	Domesticate	N. America	
***bonus-henricus* L.**	Good King Henry	Weed, wild	Eurasia	Clemants and Mosyakin (2003)
borbasii Murr.		Weed	S. America	Mulgura (1999)
bryoniifolium Bunge		Wild	Eurasia	Flora of China in http://www.efloras.org
***bushianum* var. *bushianum* Aell.**	Village goosefoot	Weed, wild	N. America	Flora of Missouri in http://www.efloras.org
***californicum* (S. Wats.) S. Wats.**	Indian lettuce, soap plant	Wild (perennial)	N. America	Clemants and Mosyakin (2003)
***capitatum* (L.) Ambrosi**	Strawberry blite	Wild	N. America	Clemants and Mosyakin (2003)
***carnosolum* Moq.**	Ridged goosefoot	Weed	S. America	Mujica and Jacobsen (2002), Mulgura (1999) and Marticorena (2010)
chaldoranicum Rahiminejad and Ghaemmaghami		Wild	Eurasia	Rahiminejad and Ghaemmaghami (2005)
***chenopodioides* (L.) Aell.**	Buttered goosefoot	Weed	S. America	Clemants and Mosyakin (2003) and Flora of China in http://www.efloras.org
cordobense Aell.		Wild	S. America	Mulgura (1999)
crusoeanum Marticorena		Wild	Juan Fernandez	Marticorena (2010)
curvispicatum Paul G. Wilson		Wild	Australia	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au
***cycloides* A. Nels.**	Sandhill goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
desertorum J.M. Black		Wild	Australia	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au
***desiccatum* A. Nels.**	Arid goosefoot	Wild, weed	N. America	Clemants and Mosyakin (2003) and Mulgura (1999)
detestans T.W. Kirk	Fishguts weed	Wild, weed	New Zealand	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au and New Zealand Plant Conservation Network in http://nzpcn.org.nz
erosum R. Br.	Papery goosefoot	Wild	Australia	PlantNet: New South Wales Flora Online at http://plantnet.rbgsyd.nsw.gov.au

(*continued*)

Table 3.1 (continued)

Species	Common name	Habit	Putative origin	References
***ficifolium* Sm.**	Fig-leaf goosefoot	Wild, weed	Asia	Clemants and Mosyakin (2003), Reiche in *http://www.efloras.org*, Flora of China in *http://www.efloras.org*, Flora of Pakistan in *http://www.efloras.org* and Marticorena (2010)
***foggii* Wahl**	Fogg's goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***foliosum* (Moench) Aschers.**	Leafy goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003) and Flora of Pakistan in *http://www.efloras.org*
***fremontii* S. Wats.**	Fremont's goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
frigidum Phil.		Wild, weed	S. America	Reiche in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
giganteum D. Don		Weed, domesticate	Eurasia	Flora of China in *http://www.efloras.org* and Mulgura (1999)
***glaucum* L.**	Oak-leaf goosefoot	Wild, weed	Eurasia	Clemants and Mosyakin (2003), Flora of China in *http://www.efloras.org*, Flora of Pakistan in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
gracilispicum H.W. Kung		Wild	Eurasia	Flora of China in *http://www.efloras.org*
halophilum		Wild	S. America	Marticorena (2010)
haumanii Ulbr.		Wild	S. America	Mulgura (1999)
***hians* Stand.**	Hians goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***hircinum* Schrad.**	Avian goosefoot, ajara	Weed	S. America	Reiche in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
***humile* Hook.**	Marshland goosefoot	Wild, weed	N. America	
hybridum L.	Maple-leaf goosefoot	Wild	Eurasia	Clemants and Mosyakin (2003) and Flora of China in *http://www.efloras.org*
iljinii Gol.		Wild	Eurasia	Flora of China in *http://www.efloras.org*
***incanum* (S. Watson) Heller**	Mealy goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
karoi (Murr) Aell.		Wild, weed	Eurasia	Flora of China in *http://www.efloras.org* and Flora of Pakistan in *http://www.efloras.org*
korshinskyi (Litv.) Minkw.		Wild	Eurasia	Flora of Pakistan in *http://www.efloras.org*
***leptophyllum* (Moq.-Tand.) Nutt. ex S. Wats.**	Narrowleaf goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
litwinowii (Paul.) Uotila		Wild	Eurasia	Flora of Pakistan in *http://www.efloras.org*
***macrospermum* Hook. f.**	Red-stemmed goosefoot	Wild, weed	S. America	Clemants and Mosyakin (2003) and Mulgura (1999)
mandonii		Wild	S. America	Marticorena (2010)
***murale* L.**	Nettle-leaf goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003), Reiche in *http://www.efloras.org*, Flora of Pakistan in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
***neomexicanum* Stand.**	New Mexico goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
nesodendron		Wild	S. America	Marticorena (2010)
***nevadense* Standl.**	Nevada goosefoot	Wild	N. America	Clemants and Mosyakin (2003)

(*continued*)

Table 3.1 (continued)

Species	Common name	Habit	Putative origin	References
nitrariaceum (F. Muell.) F. Muell. ex Benth.	Nitre goosefoot	Wild	Australia	PlantNet: New South Wales Flora Online at *http://plantnet.rbgsyd.nsw.gov.au*
novopokrovskyanum (Aell.) Uotila		Wild, weed	Eurasia	Flora of Pakistan in *http://www.efloras.org*
***oahuense* (Meyen) Aell.**	Alaweo	Wild (perennial)	Hawaii	
obscurum Aell.		Wild	S. America	Mulgura (1999)
***opulifolium* Schrad. ex Koch and Ziz**	Seaport goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003)
***overi* Aell.**	Over's goosefoot	Wild	N. America	
***pallescens* Stand.**	Light goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***pallidicaule* Aell.**	Canahua	Domesticate, weed	S. America	Mujica and Jacobsen (2002) and Mulgura (1999)
pamiricum Ijijn		Wild	Eurasia	Flora of Pakistan in *http://www.efloras.org*
papulosum Moq.		Wild, weed	S. America	Reiche in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
parodii Aell.		Wild	S. America	Mulgura (1999)
petiolare Kunth		Wild, weed	S. America	Reiche in *http://www.efloras.org*, Mujica and Jacobsen (2002), Mulgura (1999) and Marticorena (2010)
philippianum Aell.		Wild, weed	S. America	Mulgura (1999) and Marticorena (2010)
pilcomayense Aell.		Wild	S. America	Mulgura (1999)
***polyspermum* L.**	Manyseed goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003)
***pratericola* Rydb.**	Desert goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***quinoa* Willd.**	Quinoa, quingua, ajara	Domesticate, weed	S. America	Reiche in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
***rubrum* L.**	Red goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003) and Flora of China in *http://www.efloras.org*
ruiz-lealii Aell.		Wild	S. America	Mulgura (1999)
***salinum* Standl.**	Rocky Mountain goosefoot	Wild, weed	N. America	Clemants and Mosyakin (2003)
sancta-clarae Joh.		Wild (perennial)	Juan Fernandez	Reiche in *http://www.efloras.org* and Marticorena (2010)
sancti-ambrosii Marticorena		Wild	San Ambrosio and San Felix	Marticorena (2010)
scabricaule Speg.		Wild	S. America	Mulgura (1999)
***simplex* (Torr.) Raf.**	Maple-leaf goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***standleyanum* Aell.**	Standley's goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
***strictum* Roth.**	Late-flowering goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003), Flora of China in *http://www.efloras.org* and Flora of Pakistan in *http://www.efloras.org*
***subglabrum* (S. Wats.) A. Nels.**	Smooth goosefoot	Wild	N. America	Clemants and Mosyakin (2003)
suecicum Murr.		Weed	Eurasia	
trifurcatum Phil.		Wild	S. America	Reiche in *http://www.efloras.org*
***urbicum* L.**	City goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003), Flora of China in *http://www.efloras.org* and Marticorena (2010)

(*continued*)

Table 3.1 (continued)

Species	Common name	Habit	Putative origin	References
***vulvaria* L.**	Stinking goosefoot	Weed	Eurasia	Clemants and Mosyakin (2003), Flora of Pakistan in *http://www.efloras.org*, Mulgura (1999) and Marticorena (2010)
***watsonii* A. Nels.**	Watson's goosefoot	Wild, weed	N. America	Clemants and Mosyakin (2003)
***Xvariabile* Aell.**	Variable goosefoot	Weed	Hybrid	Flora of Missouri in *http://www.efloras.org*

Authoritative taxa as identified by the Integrated Taxonomic Information System (ITIS, *http://www.itis.gov/index.html*) are indicated in bold

3.2.1 Domesticated Chenopods

The most important domesticated chenopod is South American quinoa (*C. quinoa*, $2n = 4x = 36$). Quinoa is part of a complex of interfertile New World wild, weedy, and domesticated ecotypes, variously listed as three or four separate taxa. Under the system of Mosyakin and Clemants (1996), this group, from Section *Chenopodium,* Subsection *Favosa*, includes weedy South American *C. hircinum*, weedy ecotypes of North American *C. berlandieri*, and at least four extinct or surviving domesticates of *C. berlandieri*.

A second, distinct domesticated chenopod is South American *cañahua* or *cañihua* (*C. pallidicaule*, $2n = 18$). This seed crop is adapted to harsher environments than quinoa and in some respects can be considered in a state of semi-domestication (Heiser and Nelson 1974).

The last group of domesticated chenopods has its apparent origins in the wild and weedy complex of Section *Chenopodium* Subsection *Chenopodium* in Eurasia. Specifically, *C. album* is the cosmopolitan taxon of this group, and it may have been domesticated anciently in Europe, the Indian Himalayas, and China.

3.2.1.1 Chenopod Crops of the Americas

Wilson (1990) provided an extensive review of the history of *Chenopodium* cultivation in the Americas. More recent archeological evidence from the Ñanchoc Valley of northwestern Peru establishes that quinoa was domesticated sometime earlier than 7500 BP (Dillehay et al. 2007), since this region is hundreds of miles distant from the proposed centers of domestication of the crop in the Mantaro Valley or northern Altiplano (Wilson 1988b, c; Bruno and Whitehead 2003; Christensen et al. 2007). Quinoa itself existed as two distinct germplasm pools at the time of the Conquest: Andean highland quinoa (or *quinua*) with its associated weedy (*ajara*) complex; and *quingua* among the Araucanian people of the central and southern Chilean lowlands. Fuentes et al. (2009) provided simple sequence repeat (SSR)-based evidence to support coastal Chile as a second center of major quinoa diversity, either through separate domestication or, more likely, through continuous outcrossing with sympatric weedy *C. hircinum* and, since the Conquest, *C. album*. A third distinct germplasm pool, that of weedy *C. hircinum* from lowland Argentina, may represent remnants of archaic quinoa cultivation in that part of South America (Wilson 1990).

Cañahua (*C. pallidicaule*), although sympatric with quinoa, is an entirely unique domesticate capable of productivity at even higher altitudes than the latter and in soils near Lake Titicaca. The two crops are genetically isolated by their differing chromosome numbers and the cleistogamous floral structure of cañahua. Cañahua is cultivated for its mostly dark seed – small, relative to quinoa – which is primarily toasted, ground, and added to beverages to make *pito*.

The origin, or origins, of domesticated tetraploid chenopods in eastern North America is better understood than the origins of their Mesoamerican counterparts due to a profusion of archeological remains. Domesticated Mexican chenopods (*C. berlandieri* subsp. *nuttaliae*) were first described by Safford (1917), long after the Spanish Conquest, though it is highly likely that the Spanish lumped native Mesoamerican *Amaranthus* and chenopods together, referring to both simply as *bledo* (Wilson 1990). The ancient Mexicans apparently domesticated three distinct agroecotypes: a relatively common form from the

Table 3.2 Taxonomic classification of American *Chenopodium* from Aellen and Just (1943)

Section	Subsection	Species	Synonym
Roubieva		*multifidum*	*Dysphania multifidum*
		haumanii	
Orthosporum		*pumilio*	*D. pumilio*
Ambrina		*ambrosioides*	*D. ambrosioides*
Botryoides	*Botrys*	*dissectum*	
		botrys	*D. botrys*
		foetidum	
		graveolens	*D. graveolens*
	Teloxys	*aristatum*	*D. aristata*
Degenia		*frigidum*	
		chenopodioides	
		macrospermum	
Eublitum		*overi*	
		foliosum	
		capitatum	
Pseudoblitum		*mexicanum*	
		glaucum	
		rubrum	
Thellungia		*antarcticum*	
Agathophyton		*bonus-henricus*	
		californicum	
Chenopodia	*Leiosperma*	*subglabrum*	
		cycloides	
		leptophyllum	
		hians	
		papulosum	
		pratericola	
		giganteum	*C. album*
		carnosolum	
		standleyanum	
		urbicum	
		cordobense	
		fremontii	
		pilcomayense	
		petiolare	
		vulvaria	
		opulifolium	
		incanum	
		nevadense	
		pallidicaule	
		atrovirens	
		zobelii	
		albescens	*C. pratericola*
		album	
		missouriense	*C. album*
		covillei	
		strictum	
	Cellulata	*watsonii*	
		serotinum	*C. ficifolium*
		berlandieri	
		lenticulare	
		arizonicum	*C. neomexicanum*
		hircinum	
		pallescens	
		viride	*C. opulifolium*

(*continued*)

Table 3.2 (continued)

Section	Subsection	Species	Synonym
		bushianum	
		macrocalaycium	
		philippianum	
	Undata	*murale*	
		polyspermum	
	Grossefoveata	*hybridum*	
		gigantospermum	*C. simplex*

Table 3.3 Taxonomic classification of North American *Chenopodium* of Mosyakin and Clemants (1996) and Clemants and Mosyakin (2003)

Subgenus	Section	Subsection	Species	Synonym
Blitum (L.) Hiitonen	*Agathophytum*		*bonus-henricus*	
			californicum	
	Blitum (L.) Hooker fil.	*Capitata* Kowal ex Mosyakin and Clemants	*capitatum*	
		Foliosa Kowal ex Mosyakin and Clemants	*foliosum*	
	Degenia		*macrospermum*	
	Glauca		*glaucum*	*C. salinum*
	Pseudoblitum		*chenopodioides*	
			rubrum	*C. humile*
Chenopodium L.	*Chenopodium* L.	*Polysperma* Kowal ex Mosyakin and Clemants	*polyspermum*	
		Leptophylla (Standley) Clemants and Mosyakin	*atrovirens*	
			subglabrum	
			cycloides	
			pallescens	
			leptophyllum	
			hians	*C. incognitum*
			pratericola	
			desiccatum	
			foggii	
		Chenopodium L.	*vulvaria*	
			strictum	
			opulifolium	
			album	*C. giganteum*, *C. missouriense*
		Favosa (Aellen) Mosyakin and Clemants	*watsonii*	
			neomexicanum	*C. palmeri*
			ficifolium	
			berlandieri	*C. nuttaliae*, *C. bushianum*
			quinoa	
		Fremontiana (Standley) Clemants and Mosyakin	*fremontii*	
			incanum	
			nevadense	
			albescens	
		Urbica (Standley) Mosyakin and Clemants	*urbicum*	
		Undata Aellen and Iljin ex Mosyakin and Clemants	*murale*	
		Standleyana Mosyakin and Clemants	*standleyanum*	
		Grossefoveata Aellen and Iljin ex Mosyakin	*simplex*	*C. hybridum*, *C. gigantospermum*

central highlands, *huazontle*, cultivated for its broccoli-like immature panicle; a highly branched form grown for its leaves (*quelite*) in and around the Mexican state of Puebla; and *chia roja*, a brightly pigmented form grown near Lake Patzcuaro for its seeds, which are used in festive Tarascan tamales (Wilson 1990). All three form crop–weed complexes with *C. berlandieri* subsp. *berlandieri*.

There is also extensive archeological evidence for widespread chenopod cultivation from Arkansas eastward throughout eastern North America. Two distinct cultivated types of *C. berlandieri* have been identified in the archeological sites. The first, subspecies *jonesianum* Smith (Smith and Funk 1985), is classified as a domesticate because of its larger seed size and thinner enveloping epiderm, though the latter retained the shiny, black, hard outer layer like its weedy relatives. The second was morphologically identical to Mesoamerican *huazontle*, having a light brown color due to elimination of the hard outer epiderm layer, though these eastern North American strains are increasingly considered to represent an independent domestication of subsp. *nuttaliae* (Wilson 1981a, 1990; Smith 1987; Smith and Yarnell 2009). This latter type of domesticated goosefoot has been definitively identified at sites from Oklahoma in the southwest to the central Ohio Valley in the northeast, extending back to 3800 YBP (Fritz 1984; Wilson 1981a; Smith and Yarnell 2009).

Like *C. quinoa* and *C. hircinum*, the *C. berlandieri* complex is classified within subsect. *Favosa*, and North American *C. berlandieri* is considered to be the ancestor of the American allotetraploid complex that includes quinoa (Wilson 1990; Wilson and Manhart 1993). The manner of long-range dispersal from North to South America – for example, whether by migrating birds or as a follower of camps of hunting-gathering humans – is not known. However, comparative studies of isozyme and morphological variation indicated that quinoa and the *nuttaliae* crops of Mesoamerica arose from independent domestication events (Wilson and Heiser 1979; Wilson 1990).

3.2.1.2 Chenopod Cultivation in Eurasia

Lambsquarters or *C. album* is well known as a worldwide weed native to Eurasia. But there are also forms of this species that have, for millennia, been harvested mainly as a leafy vegetable, though some types are also cultivated for grain (Partap and Kapoor 1985, 1987; Joshi et al. 2002; Łuczaj and Szymański 2007). Domesticated *C. album* is an especially important crop in the Himalayan region, where it is cultivated for its seeds. The Himalayan grain chenopod is comparable to Andean quinoa in nutrient composition, and it is more nutritious than wheat, barley, maize, and rice (Partap and Kapoor 1985). Its seed protein quality equals that of milk and contains high levels of lysine (6 g/100 g protein), methionine (2.3 g/100 g protein), and cysteine (1.2 g/100 g protein). The crop is also suited to mixed farming, particularly multiple cropping systems. These domesticated Himalayan chenopods exhibit considerable morphological diversity. Four local chenopod varieties were recognized on the basis of their morphology, seed color (black, brown, red, and earthen) and uses (Partap and Kapoor 1987).

In places other than the Himalayan regions, *C. album* is mainly used as a leafy vegetable. Presently, it is being cultivated in the hill areas of North Bengal and Assam, India. Wild *C. album* (and other species such as *C. giganteum* and *C. bonus-henricus*) were/are collected and consumed in some European and Asian regions (Partap et al. 1998; Lentini and Venza 2007; Łuczaj and Szymański 2007).

The origin of domesticated *C. album* remains unexplained. The Himalayan forms of *C. album* are tetraploid ($2n = 4x = 36$) and are morphologically distinct from the weeds, which are hexaploid. Three of these varieties (black, brown, and red) appear to be correctly assigned to the *C. album* complex, whereas the earthen variety is similar to the species of subsect. *Favosa* in that it has alveolate fruits with an adhering pericarp. However, though its seeds are similar to those of quinua, the earthen variety is distinct in other respects (Partap and Kapoor 1985; Galwey 1995). The *C. album* plants cultivated as leafy vegetables in northern India are usually diploid or hexaploid (Bhargava et al. 2005).

The phylogenetic origin of hexaploid *C. album* is not well known. Gangopadhyay et al. (2002) suggested that the Indian hexaploid form is an allopolyploid and its ancestor species are two distinct varieties of diploid *C. album* (one broad leaved, another narrow leaved) along with diploid *C. murale*. The allopolyploid origin of *C. album* was supported by flavonoid evidence; however, *C. suecicum* and *C. ficifolium* were suggested as two of its ancestral diploids, since

C. murale possessed a distinct group of flavonoids not found in *C. album* (Rahiminejad and Gornall 2004).

Germplasm comprising 84 accessions of *C. album* was evaluated for 12 descriptors at the National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Shimla, India, by Joshi et al. (2002). Their evaluation revealed extensive variability in plant height; flowering time and maturity; leaf and inflorescence size; and seed yield. A wide range of variation was also reported for various other agromorphological traits. Analysis of foliage of the species of *Chenopodium* at the National Botanical Research Institute (NBRI), Lucknow, India, revealed a wide range of variation in protein (26–64 g/kg), carotene (78–190 mg/kg), vitamin C (0.5–2.4 g/kg), nitrate (2.6–5.0 g/kg), and oxalate (99–39 g/kg) contents (Joshi et al. 2002).

3.2.2 Weedy and Wild Chenopods

3.2.2.1 Distribution in North America

The Online Flora of North America (Clemants and Mosyakin 2003, *http://www.efloras.org*) provides the most comprehensive treatment of *Chenopodium* species distribution on the continent. Since the diploids native to the west are of most interest as genetic resources for improving quinoa and the Mexican domesticates, we will pay special attention to the distributions/habitats of these taxa.

The cosmopolitan weeds *C. album*, *C. berlandieri* (var. *zschackei*), *C. glaucum*, *C. murale*, *C. rubrum* (var. *rubrum*), *C. simplex*, and *C. strictum* have spread essentially throughout temperate North America. The Eurasian weeds *C. bonus-henricus*, *C. polyspermum*, *C. urbicum* are found throughout the northeast. Native *C. capitatum* is locally common in North American boreal forests and at higher elevations in the west.

The *Favosa* complex of alveolate-fruited species includes three diploids, two tetraploids, and one hexaploid. Among the diploids, *C. ficifolium* is native to Eurasia and appears sporadically as a weed in eastern North America. The native North American diploids, *C. neomexicanum* (New Mexico goosefoot) and *C. watsonii* (Watson's goosefoot), grow on disturbed acidic and alkaline soils, respectively, along the southern edge of the Colorado Plateau eastward to the Gila Mountains (Fig. 3.1). Whereas New Mexico goosefoot is locally rare in igneous ponderosa pine forests, Watson's goosefoot is more common on open grasslands, roadsides, sparse pinyon-juniper woodlands, and especially around corrals in conjunction with mealy goosefoot (*C. incanum*). It has also been reported, though less commonly, from the western Great Plains in New Mexico northward to Wyoming. The two species are easily distinguished phenotypically: New Mexico goosefoot by its relatively glabrous triangular leaves and upright growth habit and Watson's goosefoot by its gray or blue–green, farinose, highly fetid-smelling foliage and highly branched growth habit.

Berlandier's, or pitseed, goosefoot (*C. berlandieri*) is a common weed of roadsides, construction sites, stream banks, and other disturbed locales throughout the continent, as far north as Alaska (Fig. 3.2). Clemants and Mosyakin (2003) subdivided this species into an assortment of botanical varieties within subspecies *berlandieri*: *berlandieri* (Texas); *boscianum* (Gulf coast and lower Great Lakes); *bushianum* (croplands and abandoned fields of the Mississippi and Ohio Valleys); *macrocalycium* (northeast coast); *sinuatum* (southwest); and *zschackei* (widespread across most of the continent west and northwest of the Mississippi and Ohio Rivers). Of these botanical varieties, *macrocalycium* is noteworthy for its relatively large (2+ mm) fruits. In addition, subspecies *nuttaliae*, which encompasses the native Mexican agroecotypes, is commonly recognized. Clemants and Mosyakin (2003) noted that *C. quinoa*, which is occasionally cultivated in gardens and commercially in higher valleys of the Rocky Mountains, keys to *C. berlandieri* in their taxonomic treatment. Cytological studies (Kolano unpublished) have verified that var. *bushianum* is hexaploid with 54 chromosomes and sequencing of nuclear genes (Jellen unpublished) has verified that this taxon carries at least one unique subgenome when compared to the phylogenetically proximal allotetraploid complex that includes subsp. *nuttaliae*, *C. quinoa*, South American *C. hircinum*, and *C. berlandieri* vars. *zschackei* and *macrocalycium*. We have concluded that morphological similarities, particularly for the alveolate testa, likely derive from all these species sharing a common subsect, *Favosa* diploid ancestor. These results also suggest that *bushianum* should be classified as a separate species.

The *Leptophylla* complex of narrow-leaved species includes nine North American diploids (Fig. 3.3).

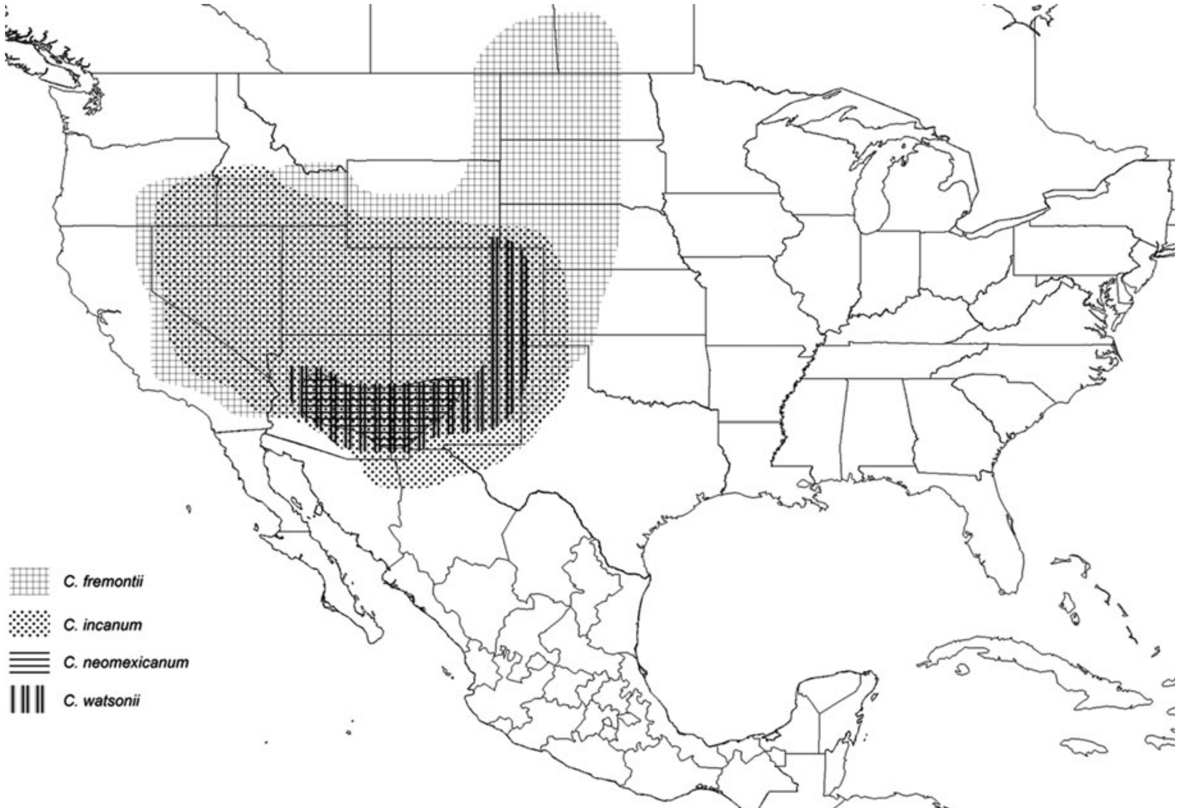


Fig. 3.1 Geographic distribution of North American *Chenopodium* diploids that are apparently most closely related to the 4*x* *C. berlandieri*–*C. hircinum*–*C. quinoa* complex. *C. fremontii* and *C. incanum* have smooth utriculate fruit and belong to Subsection *Fremontiana*. *C. neomexicanum* and *C. watsonii* have alveolate achenes and are assigned to Subsection *Favosa*. Adapted from Clemants and Mosyakin (2003) and personal observations

These are small, inconspicuous herbaceous plants, which are either mildly aggressive weeds of disturbed sandy soils (*C. desiccatum* and *C. pratericola*) or episodic in appearance in discrete niche habitats. Of these, the most widespread are narrowleaf goosefoot (*C. leptophyllum*), which is rare but native to deserts and sandy soils throughout the western third of the continent, and the more common *C. pratericola*, which grows on disturbed, sandy soils from California eastward into the Mississippi and lower Ohio Valleys. Mountain or pinyon goosefoot (*C. atrovirens*) is locally common on disturbed soils in the central Rocky Mountains and Great Basin. Aridland goosefoot (*C. desiccatum*) is locally common, but episodic in appearance at middle elevations on sandy soils from the Great Basin spreading eastward into the Great Plains. Hians' goosefoot (*C. hians* formerly known as *C. incognitum*) is locally common, but can be episodic in seasonally drier montane and foothill arroyos from the Peninsular and Transverse Ranges of southern California northward through the foothills of the Sierra Nevada and eastward to the Gila Mountains of New Mexico. The sandhill goosefoots (*C. cycloides* and *C. subglabrum*) are locally rare, appearing sporadically in sandy areas like stream banks, blowouts, moist depressions, and stabilized dunes from the Texas Panhandle northward through the Sand Hills of Nebraska to the Saskatchewan–Alberta border region. Further study is probably needed to determine if *C. cycloides* qualifies for federal protection as an endangered species. Pallid goosefoot (*C. pallescens*) is native to the southern and central Plains on sandy prairies to open woodlands. Fogg's goosefoot (*C. foggii*) is locally rare on forest soils of the northeastern United States. Certain of these taxa can be extremely difficult to differentiate, with discriminating morphological criteria including the presence of one midvein (*C. cycloides*, *leptophyllum*, *C. pallescens*, *C. subglabrum*) versus three leaf veins (*C. atrovirens*, *C. desiccatum*, *C. foggii*, *C. hians*, *C. pratericola*); adhering pericarp as an achene

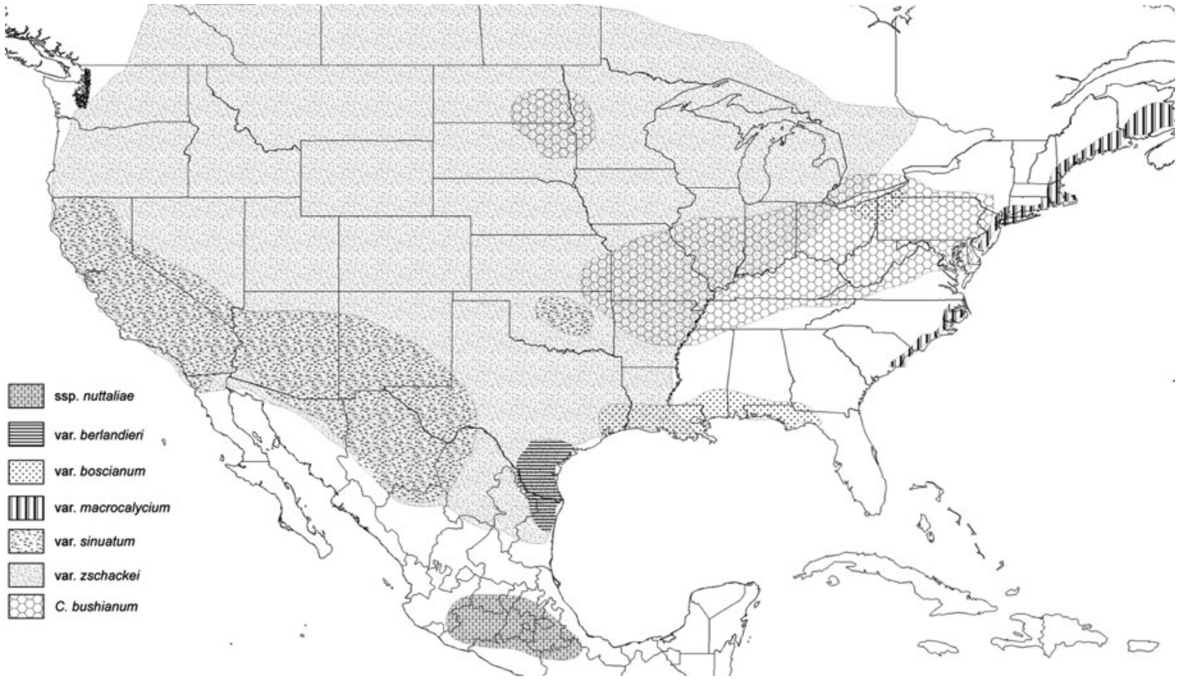


Fig. 3.2 North American distribution of various forms of allotetraploid *C. berlandieri* and allohexaploid *C. bushianum* (syn. *C. berlandieri* var. *bushianum*). Adapted from Clemants and Mosyakin (2003) and personal observations

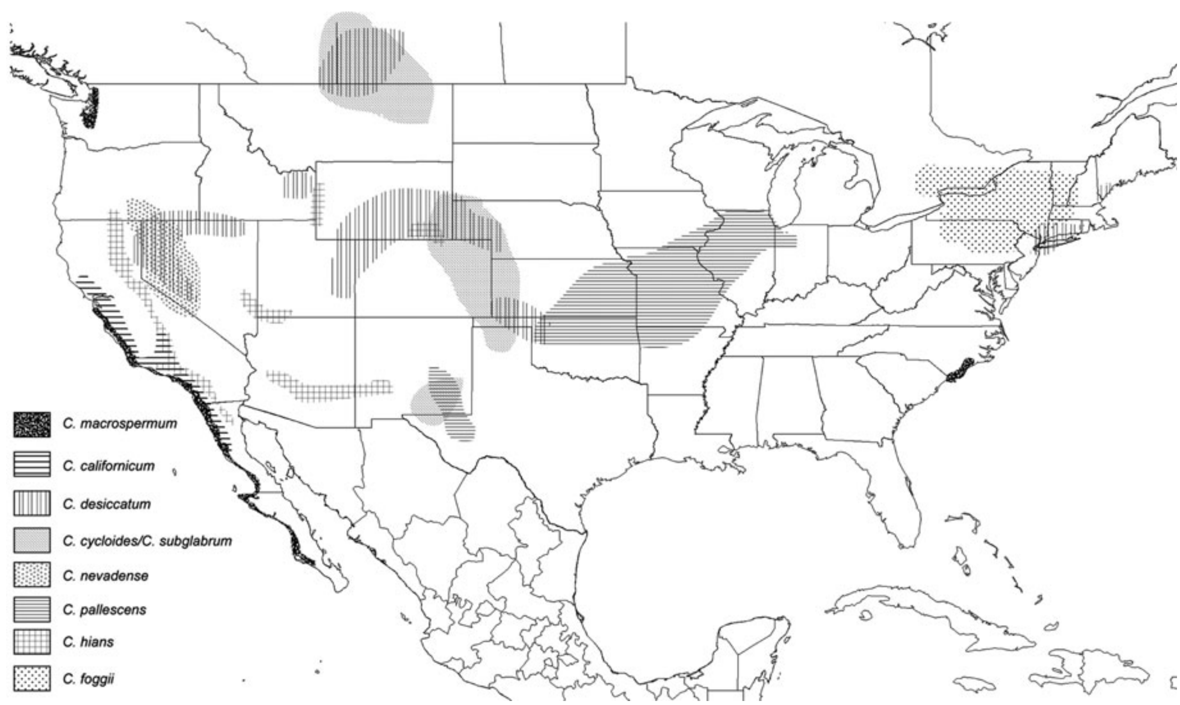


Fig. 3.3 Biogeography of various locally distributed North American *Chenopodium* diploids and South American-native tetraploid *C. macrospermum*. Subsection *Leptophylla* diploids not shown include *C. atrovirens*, *C. leptophyllum*, and *C. pratericola* because these taxa are widely distributed throughout the interior west. Adapted from Clemants and Mosyakin (2003) and personal observations

(*C. cycloides*, *C. hians*, *C. leptophyllum*, *C. pallescens*) versus utriculate pericarp (*C. desiccatum*, *C. pratericola*, *C. subglabrum*); inflorescence type; stem growth habit (erect versus spreading, basally versus distal branching); and farinose (*C. atrovirens*, *C. desiccatum*, *C. foggii*, *C. hians*, *C. leptophyllum*, *C. pratericola*) versus glabrate (*C. cycloides*, *C. pallescens*, *C. subglabrum*) leaf and stem surfaces.

The *Fremontiana* group of North American diploids includes four North American species: Fremont's goosefoot (*C. fremontii*), silvery or mealy goosefoot (*C. incanum*), Nevada goosefoot (*C. nevadense*), and *C. albescens*. Fremont's goosefoot is relatively common in dry but shady habitats, and especially in pinyon-juniper woodlands, from the Sierra Nevada and Transverse Ranges of California eastward to the Great Plains (Fig. 3.1). The other widespread but episodic species of this group, mealy goosefoot, is usually found in disturbed, open habitats like arroyos and roadsides within the southern Great Basin, Mojave, Colorado Plateau, and Chihuahuan deserts, or along roadsides and other disturbed areas in the western Great Plains. Three botanical varieties were recognized in this species by Crawford (1977): var. *incanum* (western Great Plains); var. *elatum* (southern Arizona–New Mexico); and var. *occidentale* (Great Basin and Mojave Desert). While *C. incanum* tends to be short, highly basally branched, and inconspicuous with its gray-green, highly farinose foliage, *C. fremontii* has an erect growth habit and large, triangular, light green leaves with prominent basal lobes. Nevada goosefoot is very episodic, though locally abundant in favorable years, on highly alkaline soils in the western Great Basin. Similar to *C. incanum*, it is short, inconspicuous, and highly branched at the base. The two species are distinguished by their fruit morphology (utricle in mealy goosefoot versus achene in Nevada goosefoot) and leaf texture (highly farinose in mealy goosefoot and mostly glabrous in Nevada goosefoot). *C. albescens* is a highly localized and poorly defined taxon from southern Texas. Neither Subsection *Fremontiana* nor Subsection *Leptophylla* includes identified polyploids.

Other taxa native to western North America include the diploids *C. californicum*, *C. humile*, *C. simplex*, and *C. salinum*, along with the tetraploid *C. macrospermum*. These species are particularly interesting as genetic resources for improving quinoa due to their biochemical, morphological, and/or adaptive characteristics. Indian lettuce or soap plant (*C. californicum*) is endemic to the California Floristic Province. Its two interesting features are perennial growth habit and a large taproot that accumulates triterpenoid saponins (hence its use by indigenous California tribes as a source of soap). Low goosefoot, *C. humile* (International Taxonomic Identification System [ITIS] designation, also known as *C. rubrum* var. *humile*), and the mostly sympatric Rocky Mountain goosefoot *C. salinum* (ITIS designation, also *C. glaucum* var. *salinum*), are low-growing forbs found along moist saline and alkaline shorelines in the Great Basin and other brackish soils of the west. Of prime interest for its salinity tolerance is red-stemmed goosefoot, *C. macrospermum* (Fig. 3.4), an estuarine species especially abundant in coastal California but thought to have spread northward from South America. This species, along with maple-leaf goosefoot (*C. simplex*) and Indian lettuce, is also of potential interest for quinoa improvement due to its large seed size.

3.2.2.2 Distribution in South America

Chenopod distribution in South America is primarily restricted to highland Andean, subtropical, and temperate regions of the continent. The most common species are Eurasian exotics, especially *C. album* and *C. murale*, along with the native New World wild/weedy/domesticated *C. hircinum*–*C. quinoa* (*C. berlandieri*) complex. At least three factors complicate the identification and classification of these common chenopods. The first is the systematic separation of *C. hircinum* and *C. quinoa* (and *C. berlandieri*), which is taxonomically problematic since it is based largely on growth habit (weedy versus domesticated, respectively) and ecological range (eastern Pampa versus Andean/Pacific), rather than cross-incompatibility (Aellen and Just 1943; Wilson 1981b, 1988a). The second is phenotypic plasticity for confounding morphological characters and, in the case of *C. album*, the presence of diploid, tetraploid, and hexaploid forms. The third problematic factor is limited genetic, combined with abundant anecdotal, evidence for interspecific hybridization in the field, particularly in Chile, between *C. quinoa* and weedy *C. hircinum* and sympatric *C. album* (presumably tetraploid forms or misclassified 4*x* *C. strictum*; personal observations of the authors and I. von Baer; Fuentes

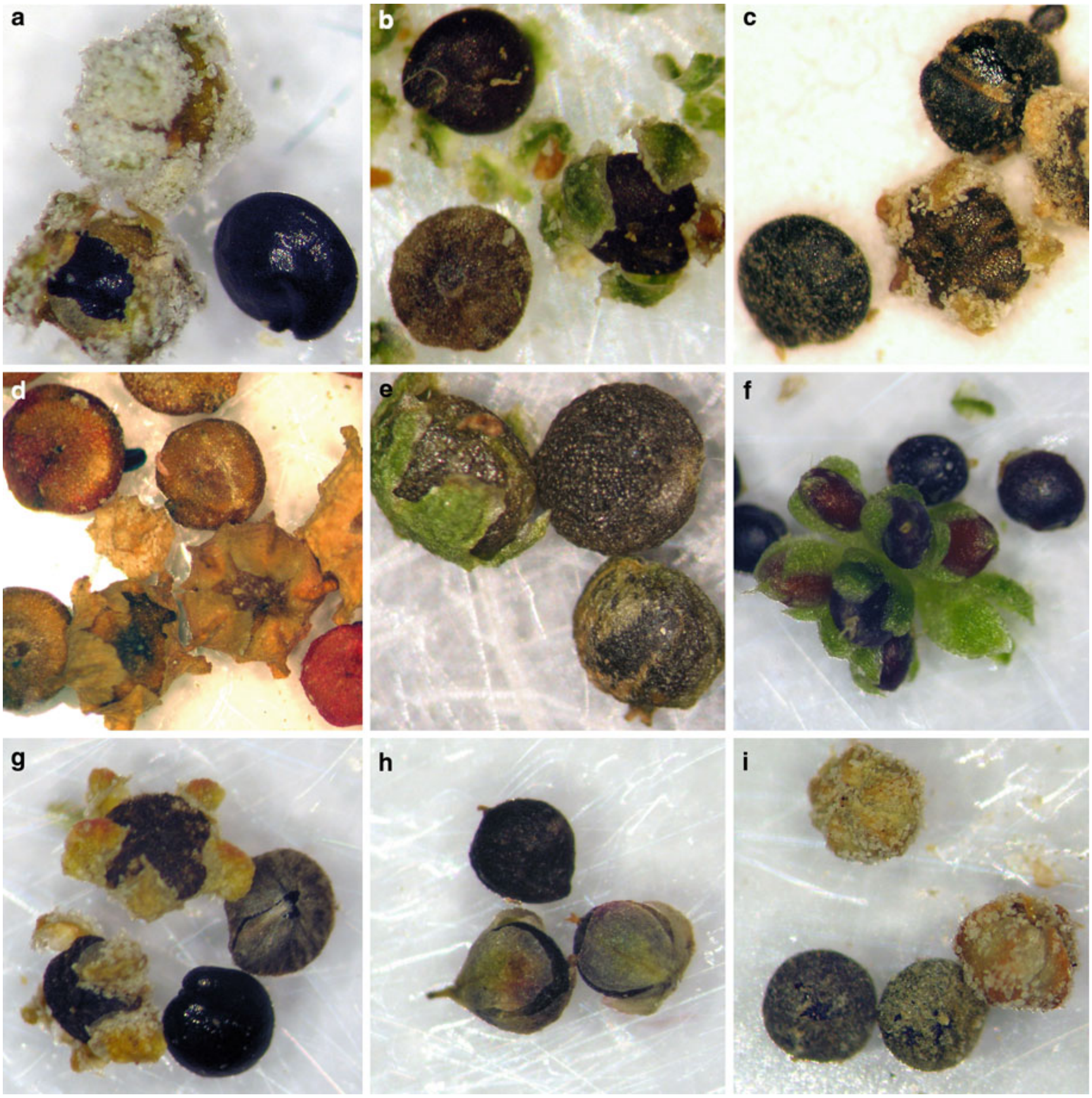


Fig. 3.4 Photographs of representative *Chenopodium* species: (**a**) *C. album* in an urban alley, Sale, Morocco; (**b**) *C. murale* (*arrowhead*) and *C. vulvaria* (*arrow*) on clay in a rock outcrop, Ourika Valley, Morocco; (**c**) *C. strictum* on Mekong River alluvium, Chiang Saen, Thailand; (**d**) *C. macrospermum* on irrigated sand in Iquique, Chile; (**e**) *C. frigidum* along the roadside in the precordillera of Tarapaca, Chile; (**f**) *C. pallidicaule* experimental plots at Quipaquipani, Bolivia; (**g**) *C. fremontii* on clay loam in shade of *Quercus gambelii* on Mt. Timpanogos, Utah; (**h**) *C. incanum* var. *occidentale* on previous-year burn area near Cove Fort, Utah; (**i**) *C. atrovirens* on limestone substrate behind Squaw Peak, Utah; (**j**) *C. watsonii* (*arrowhead*) and *C. berlandieri* var. *sinuatum* (*arrow*) in pinyon-oak-shrub steppe near Ash Fork, Arizona; (**k**) *C. nevadense* on black gravel alkali pavement near Fallon, Nevada; (**l**) *C. neomexicanum* in pine woodland near Payson, Arizona

et al. 2009). In irrigated portions of the Atacama Desert of northern Chile, for example, we have noted sympatric weedy populations of *C. album* and *C. hircinum*, distinguishable from each other by seed morphology (smooth versus alveolate, respectively) and DNA sequence variants (cpDNA and nuclear *SOS1* intronic sequences, Jellen and Fuentes unpublished). Cytological examinations of specimens from highland Peru, previously identified locally by plant morphology as diploid *C. petiolare*, had $2n = 6x = 54$ chromosomes

and were therefore possibly also exotic *C. album*. The diploid weed *C. murale*, which is a native of Eurasia, is locally common at middle to low elevations of the central Andes, on irrigated soils of the western coastal desert belt and subtropical Brazil, extending southward into central Chile and the Uruguayan–Argentine Pampas into Patagonia (Mulgura 1999; *http://www.efloras.org*).

Several native South American species are also relatively common. Red-stemmed goosefoot (*C. macrospermum*) is a highly polymorphic diploid (Jellen unpublished; *http://www.efloras.org*) and/or tetraploid (Giusti 1970) in littoral to estuarine habitats, having been observed along the Pacific coast at Miraflores, Peru, and in Iquique, Chile (Fig. 3.4; Jellen unpublished), in addition to the Argentine Atlantic lowlands (Mulgura 1999).

Other *Chenopodium* species that are locally common to rare include the economically important high-altitude pseudocereal *cañahua* or *cañihua* (*C. pallidicaule*, Fig. 3.4), a series of weedy highland diploids,,coastal populations of *C. macrospermum*, and several taxa native to the southeastern lowlands. *Cañahua* is a localized subsistence seed crop in Peru and Bolivia, mainly on the Altiplano and elsewhere at altitudes exceeding 3,600 meters (Risi and Galwey 1984). *C. carnosolum* exists as multiple weedy ecotypes in flooded and saline habitats around Lake Titicaca (Mujica and Jacobsen 2006) and extends southward through the Altiplano and into the higher valleys of northern Argentina and Chile (Mulgura 1999). *C. petiolare* is described as a common diploid weed in traditional cultivation fields around Lake Titicaca (Mujica and Jacobsen 2006), extending throughout the Altiplano and valleys of the central Andes (Mulgura 1999), characterized by a similar phenotype to quinoa but with shattering, black seed and tending toward a single, erect stem, and having excellent drought tolerance. We (Jellen, Maughan and Fuentes unpublished) have observed locally common, weedy populations of *C. petiolare* along roadsides and in towns of the Chilean Cordillera Occidental at elevations exceeding 3,000 m. *C. incisum* is reported to be sympatric with *C. petiolare* and *C. carnosolum* in the Lake Titicaca region, though its placement in *Chenopodium*, as opposed to *Dysphania*, may be in question due to morphological similarities with *D. graveolens* (Mulgura 1999; Mujica and Jacobsen 2006). *C. philippianum* and *C. frigidum* are also reported from the higher central–southern Andes (Mulgura 1999; Mujica and Jacobsen 2006). Wild species distributed primarily within the southeastern lowlands or Patagonia include *C. burkhartii*, *C. cordobense*, *C. obscurum*, *C. papulosum*, *C. parodii*, *C. pilcomayense*, *C. ruiz-lealii*, and *C. scabricaule* (Aellen and Just 1943; Mulgura 1999). Several novel taxa reported in the Chilean flora (Marticorena 2010; *http://www.chlorischile.cl*) include *C. macrocarpum*, *C. halophilum*, and *C. nesodendron*, along with species that most likely belong to *Dysphania* in the more recent classification system (Fig. 3.5).

There are a number of other wild/weedy plants formerly classified as *Chenopodium* and more recently in the genus *Dysphania* that are common and, in some cases, economically important in South America. The most widespread and important of these is *paico* or *D. ambrosioides*, a locally important medicinal plant in the Andes.

3.2.2.3 Distribution in Eurasia and North Africa

The best known Eurasian species is *C. album*. It is almost cosmopolitan, being common in subtropical to temperate zones and less frequent in the tropics. This species' center of diversity is likely in the Himalaya Mountains (Partap et al. 1998). Today, it is one of the most important and difficult weeds to control, being very common in gardens, abandoned fields, construction sites, roadsides, stream banks , irrigated places, and other ruderal areas. It is also reported as a halophytic plant in some parts of Asia (Brenan and Akeroyd 1992; Tanaka and Tanaka 1980).

A number of other chenopods grow in disturbed habitats and it is often impossible to decide whether they fall into the category of passively established alien versus native in a given region. The *Chenopodium* species *C. ficifolium*, *C. strictum*, *C. murale*, and *C. botrys* are widely distributed in Europe, Asia and North Africa. We (Jellen and Maughan unpublished) have observed isolated *C. strictum* plants along the bank of the Mekong River in tropical Thailand at 20.35°N latitude, far downstream from its "native" range in central and western China – obviously a consequence of seed dispersion by seasonal alluvial flooding (*http://www.efloras.org*; Fig. 3.4). Others, like *C. glaucum* and *C. hybridum*, are distributed in most of Europe and Asia between 40°N and 60°N (Meusel et al. 1978; Brenan and Akeroyd 1992).



Fig. 3.5 Variation for fruit morphology in *Chenopodium*: (**a**) *C*. *incanum* with utriculate fruits and semi-keeled sepals (Fivemile Pass, Utah); (**b**) *C*. *nevadense* achenes (Wilson Canyon, Nevada); (**c**) *C*. *leptophyllum* achenes (Ephraim Canyon, Utah);

The more southerly ranging *C. foliosum* is found from the western Mediterranean to the Himalayas and the Altai Mountains; it is thought to have originally been native to high altitudes, but anciently became naturalized within its current range after being brought into cultivated places like gardens and in other disturbed sites (Uotila 1993). Common weedy species in the Maghreb, besides *C. album,* include *C. opulifolium*, *C. murale*, and *C. vulvaria* (Fig. 3.4).

Not all Eurasian *Chenopodium* species have such a wide range. For example, good King Henry (*C. bonus-henricus*) is distributed mainly in Europe, where it is cultivated as a leafy vegetable and has spread as a weed to nitrate-rich soils in the countryside. Another local weed, *C. karoi*, is found in the mountains, fields, and around farms and wastelands of central and East Asia. Other examples of locally distributed species include *C. litwinowii* in the Pamirs and Hindukush Mountains at altitudes of 2,300–4,300 m and *C. exsuccum* in Spain and N.E. Portugal (Uotila 1981, 1993; *http://www.efloras.org* 2008).

Apart from the native species, there are also several naturalized chenopods in Eurasia including *C. berlandieri*, *C. bushianum*, *C. capitatum*, and *C. chenopodioides*. Some of them were initially cultivated in gardens as herbs or ornamentals but have since spread to ruderal and other disturbed roadsides, stockyards, railroad frontages, etc. (Brenan and Akeroyd 1992; *http://www.efloras.org* 2008). Other species spread into Europe via contaminated wool from sheep-producing regions like Patagonia (i.e. *C. hircinum*) and Australia (i.e. *C. nitrariaceaum*; Stace 1997).

3.2.2.4 Distribution in Australia and Oceania

Australia hosts unique *Chenopodium* and *Dysphania* biodiversity, along with the globally distributed exotic weeds like *C. album*, *C. glaucum*, *C. murale*, and *C. vulvaria*. Native Chenopodiaceae seeds were important as a gathered food source as evidenced by remains in rock shelters of northwestern Australia from 40,000 YBP (McConnell 1998). Many of the native *Chenopodium* species of Australia were recently transferred to *Dysphania* (Shepherd and Wilson 2008). Species that remain in the genus include *C. auricomiforme*, *C. auricomum*, *C. curvispicatum*, *C. desertorum*, *C. detestans*, *C. erosum*, and *C. nitrariaceaum* (Table 3.1; New South Wales Flora Online, *http://plantnet.rbgsyd.nsw.gov.au*). Although not domesticated, these mostly perennial species are considered to be important range forage on saline-sodic soils. The New Zealand–native fishguts weed (*C. detestans*) has also become naturalized as a weed within southeastern Australia (New South Wales Flora Online).

In addition to these species, *Chenopodium* species are native to three distant archipelagoes of the Pacific Ocean. Alaweo (*C. oahuense*) is a perennial shrub endemic to the Hawaiian Islands. *Chenopodium sancti-ambrosii* is reportedly endemic to the Desventuradas Islands approximately 900 km off the Chilean coast just south of 26°S latitude (Marticorena 2010). Two additional species, *C. crusoeanum* and *C. sanctaclarae*, are endemic to the Juan Fernandez Islands (Marticorena 2010).

3.2.3 Germplasm Conservation

3.2.3.1 Genetic Erosion in Quinoa

Quinoa and cañahua production and acreage suffered a tremendous post-Conquest decline, which actually accelerated during the interval between 1947 and 1975 (Tapia 1979). This acceleration in abandonment of the native crops in Peru and Bolivia was attributed to import subsidies, climatic factors, and cultural disparaging of "Indian" crops and culture (Tapia 1979; Risi and Galwey 1984). Fortunately, this situation has completely reversed itself, with Andean (Bolivia–Peru) quinoa production increasing dramatically in order to attempt to keep pace with increasing domestic and export demands for high-quality quinoa seed: from ~31,600 tons in 1961, to a low of ~14,800 tons in 1968, to a high of ~54,600 tons in 2007 (UN-FAO statistics, *http://faostat.fao.org*). Harvested quinoa

Fig. 3.5 (continued) (**d**) *C. berlandieri* ssp. *nuttaliae* (cultivated *huazontle*) pseudograin achenes (Colima, Mexico); (**e**) *C. californicum* achenes (Fallbrook, California); (**f**) *C. capitatum* with vertical achenes and 3-sepaled flowers (Payson Canyon, Utah); (**g**) *C. desiccatum* utricles with spreading sepals (Maybell, Colorado); (**h**) *C. humile* with connate sepals and vertical achenes (Utah Lake, Utah); (**i**) honeycombed achenes with alveolate pericarps in *C. watsonii* (Peach Springs, Arizona)

acreage in the two countries concomitantly increased during the same time interval from ~50,600 ha (1961) to a minimum of ~27,700 ha (1979) up to ~71,500 ha in 2007 (*http://faostat.fao.org*).

History, both natural and political, suggests superficially that quinoa may have passed through at least three genetic bottlenecks, though on closer scrutiny these may not have resulted in a tremendous loss of genetic diversity. The first, and potentially most severe, genetic bottleneck would have occurred when the two diploid ancestors of quinoa went through their hybridization/chromosome doubling event, assuming: (1) that this resulted in a reproductively isolated allotetraploid species (most likely *C. berlandieri*; Wilson 1990) and (2) that this event occurred only once, meaning that the 4*x* New World species complex had a monophyletic origin.

The second putative bottleneck would have occurred when quinoa was domesticated from its wild/weedy tetraploid ancestor; however, this genetic constriction may not be so significant given quinoa's ability to outcross with other 4*x* species (Wilson and Manhart 1993) and the fact that it is sympatric with multiple wild/weedy 4*x* forms of *C. hircinum* and/or *C. quinoa* var. *melanospermum* (Wilson 1981b; Mujica and Jacobsen 2006). The significance of this second genetic bottleneck is directly dependent upon the first, since monophylogeny of the 4*x* complex implies the presence of relatively little genetic diversity for exchange in its cross-compatible wild relatives. Another possibility, recently borne out by DNA marker diversity studies, is that quinoa was domesticated twice: once in the High Andes and a second time in the Chilean lowlands (Christensen et al. 2007; Fuentes et al. 2009).

The third, political bottleneck happened over the span of 400+ years from the Conquest to the 1980s, during which time quinoa was marginalized for cultural reasons. There is abundant evidence that throughout quinoa's original range at the time of the Conquest, the crop has since been shifted onto marginal lands that are less suitable for cultivation of exotic, salt- and drought-sensitive crops like wheat (Risi and Galwey 1984; Lescano 1994). Risi and Galwey (1984) identified five original ecotypes of quinoa: (1) Valley; (2) Altiplano; (3) Salar (southern Altiplano); (4) Sea Level (Coastal Chilean); and (5) Subtropical or Yungas. Of these groups, the Yungas ecotypes are the least collected and characterized, by far.

Of the five quinoa ecotypes of Risi and Galwey (1984), the most susceptible to genetic erosion are the Valley and Sea Level groups. As they pointed out, much of the quinoa cultivation in northern Andean Valley regions has been supplanted by cash or exotic crops; here, quinoa farmers on more productive alluvial soils have been persuaded to replace lower yielding landraces with higher yielding cultivars like "Ingapirca" (Ecuador), "Rosa de Junin," and "Blanca de Hualhuas" in the Mantaro Valley of Peru under mechanized conditions. Exploratory collection efforts in recent years in the Callejon de Conchucos, Cusco, Cajamarca, and Apurimac valleys of Peru by scientists at the National Agrarian University of La Molina (UNALM) uncovered the existence of apparently extensive phenotypic variation (Gomez personal communication). Within the Chilean lowlands, small-scale indigenous Mapuche farmers continue to cultivate heterogeneous quinoas for mostly local consumption, while production in areas north of the Rio Biobio is becoming increasingly commercialized with cultivars like "Regalona," "Faro," and "Pichilemu" (Risi and Galwey 1984). On the other hand, the Chilean lowlands have apparently witnessed considerable gene exchange between quinoa and sympatric weeds that has diversified the cultivated gene pool (Fuentes et al. 2009).

Rojas (2003) and Christensen et al. (2007) identified abundant variation for morphological characters and microsatellite markers, respectively, in Bolivian quinoa germplasm. The vast majority of the genotypes examined in both studies were Altiplano and Salar ecotypes. Within these regions, quinoa breeders with the Foundation for the Promotion and Investigation of Andean Products (PROINPA, Bolivia) and the National University of the Altiplano–Puno (UNAP, Peru) have consciously endeavored to develop quinoa cultivars derived from a broad genetic base. In addition, agronomists working in the northern Altiplano, a region characterized by small subsistence farms and tremendous quinoa diversity, have encouraged them to retain heterogeneous traditional cultivation systems like the *aynoka* (Mujica and Jacobsen 2006). The Salares, in contrast, are characterized by dryland, mechanized, cooperative quinoa farms extending across huge acreages on very marginal soils, with much of the crop being harvested for export through the rail terminal at Uyuni, Bolivia. Here, a heterogeneous assemblage of large- and white-seeded "Real"

varieties have been developed, literally through hundreds of years of selection, including strains such as "Kellu," "Lipeño," "Achachino," "Hilo," "Toledo," variegated "Sayaña," and "Ollagüe" (Chilean).

3.2.3.2 Genetic Erosion in Wild and Weedy *Chenopodium* Species

The goosefoot genus is a paradoxical mix of seriously threatened, ephemeral endemics and some of the world's most notorious cosmopolitan weeds. Obviously, universal weeds like *C. album*, *C. berlandieri* var. *zschackei*, and *C. murale* that have thrived and proliferated in the presence of anthropogenic disturbance are not susceptible to genetic erosion. On the other hand, two botanical varieties of *C. berlandieri–boscianum* and *macrocalycium* along with *C. album* var. *missouriense* are listed as protected by various state governments (*http://plants.usda.gov*). Other ephemeral, wild species that are threatened or endangered in North America include *C. cycloides*, *C. standleyanum*, and *C. foggii*. The wild species endemic to Argentina and rare Eurasian species like the newly described *C. chandoranicum* might also be threatened or endangered. The Pacific archipelago endemics *C. crusoeanum*, *C. oahuense*, *C. sancta-clarae*, and *C. sancti-ambrosii* are the species expected to be in greatest danger of erosion due to habitat loss and climate change.

3.2.3.3 Ex Situ Conservation Efforts

The most extensive ex situ germplasm collections for *Chenopodium* are maintained by the Royal Botanic Garden at Kew, the USDA-ARS, the National Bureau of Plant Genetic Resources (India), PROINPA (the Bolivian national collection), UNAP, and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany). Of these gene banks, only the USDA-ARS and Royal Botanic Garden at Kew maintain significant collections of wild *Chenopodium* native to the Americas.

The primary characterized gene bank in Peru is held by UNAP in Puno, which reported a collection of 1,029 quinoa accessions, from which they defined a core collection of 103 (Ortiz et al. 1998, 1999). These authors reported the greatest amount of variation in Peruvian material from the Altiplano, though their collection was heavily biased toward accessions from the Puno and Cusco regions (742/1,029 or 72%). The collection did not contain wild species. Other Peruvian research groups also maintain gene banks, for example the UNALM, with a collection of approximately 2,800 quinoa and 140 cañahua accessions to date, including collections from central and northern Peru (Gomez personal communication).

The PROINPA Foundation was granted custody of the Bolivian national quinoa collection in the late 1990s. They actively collect and maintain their germplasm in an ex situ collection facility recently constructed at Quipaquipani 4 km south of Viacha, Ingaviri Province, in the northern Altiplano. Collection efforts are ongoing, with approximately 3,120 quinoa (282 in the core collection) and 770 canahua accessions in custody at the site. They also have collected 67 accessions of *paico* (*Dysphania ambrosioides*).

The USDA-ARS collection is maintained within the National Plant Germplasm System (NPGS) at Ames, Iowa (location NC-7). It currently consists of 137 quinoa, 13 cañahua, 13 *C. album*, eight *C. giganteum*, five weedy *C. berlandieri*, four domesticated *C. berlandieri nuttaliae*, two *C. capitatum*, two *C. foliosum*, two *C. murale*, and single accessions each of *C. bonus-henricus*, *C. bushianum*, *C. ficifolium*, *C. fremontii*, *C. glaucum*, *C. neomexicanum*, *C. rubrum*, *C. simplex*, *C. standleyanum*, *C. strictum*, and *C. vulvaria*. However, this collection is currently being updated with additional accessions of wild/weedy *C. atrovirens*, *C. berlandieri*, *C. californicum*, *C. desiccatum*, *C. fremontii*, *C. hians*, *C. humile*, *C. incanum*, *C. leptophyllum*, *C. macrospermum*, *C. neomexicanum*, *C. nevadense*, *C. salinum*, *C. strictum*, and *C. watsonii* recently collected by the authors in the western United States.

In addition to the American ex situ gene banks are several known collections in Europe and India. Risi and Galwey (1989) reported morphological data on a collection of 294 quinoa accessions at the Cambridge University. In addition, the Royal Botanic Gardens at Kew, as part of the Millennium Seed Bank Project, maintains what is probably the broadest collection, consisting of 57 species, at least 11 of which are now classified in the genus *Dysphania* and 18 of which are native to the New World. Partap et al. (1998) reported the existence of collections of 99 and 18 mostly

C. album and *C. amaranticolor* (syn. *C. giganteum*) accessions at the National Bureau of Plant Genetic Resources (NBPGR) at Shimla, India, and the National Botanical Research Institute (NBRI) at Lucknow, India, respectively, which are the fruit of extensive collection efforts in the *Chenopodium* production areas of the Himalayas. Additionally, the IPK-Gaterseleben gene bank in Germany has an extensive collection of 1,044 available accessions, mostly Eurasian species and quinoa.

Ex situ collections of wild *Chenopodium* are fairly easy to maintain but difficult to use because of seed dormancy. We have observed that different species, and even genotypes within a species, respond differently to the same germination protocol. We have used variations ranging from 1 to 30 days at 4°C in 50–500 ppm GA_3, usually following a scarification procedure involving chipping of the enamel-like seed coat using a scalpel. In general, a 3-day treatment in 500 ppm GA_3 at room temperature works best. In addition, some genotypes are extremely susceptible to damping off as seedlings and must be protected with a fungicide until the plant is fully established at the four-leaf stage. A seed germination technique described by Hock et al. (2006) involving cold stratification and solid matrix priming (SMP®) in *C. album* also appears very promising.

3.2.3.4 In Situ Germplasm Conservation

In situ quinoa germplasm conservation, including farmer-participatory breeding, remains an important focus of quinoa improvement programs in the Andean region (Danielsen et al. 2000; Mujica and Jacobsen 2006; McElhinny et al. 2007). Tapia (2000) reviewed an exemplary, tri-entity approach to in situ conservation in Cajamarca Department, Peru, involving the International Potato Center (CIP), the Association for Rural Development in Cajamarca (ASPADERUC), and the Consortium for the Sustainable Development of the Andean Ecoregion (CONDESAN). The six steps of the model are: (1) soil and water conservation and management; (2) community seed fairs highlighting biodiversity, rather than best-performing lines; (3) farmer-participatory workshops; (4) farmer-organized conservation associations; (5) establishment of seed banks on farms having the greatest diversity; and (6) periodic visits of oversight institutions to monitor progress in maintaining biodiversity. Similar projects have been underway in central Mexico since 2004 to actively promote in situ conservation of native *C. berlandieri* subsp. *nuttaliae* landraces, many of which are on the brink of extinction (Perez-Agis et al. 2005).

Partap et al. (1998) and Partap and Kapoor (1985) pointed out the urgent need for in situ conservation to prevent genetic erosion in the Himalayan cultivated chenopods. Partap and Kapoor (1985) reported that remnant chenopod cultivation is strongly correlated with low socioeconomic status, low income, and socially conservative cultural practices – as was the case in the Andean region until recently. Partap et al. (1998) identified 14 areas in India, the Guizhou Plateau in China, and north-central Bhutan as regions where remnant chenopod cultivation merits further collection and in situ conservation efforts.

In contrast to the cultigens, wild goosefoot conservation is being widely ignored and is therefore a matter of utmost concern, there being a common misperception that *Chenopodium* is a genus strictly of noxious weeds such as lambsquarters (*C. album*), nettle-leaved goosefoot (*C. murale*), and pitseed goosefoot (*C. berlandieri*). Within southwestern North America – the area most intensively surveyed by the authors – diversity-rich, multispecies goosefoot communities present one year will be missing for two or more subsequent years, depending upon seasonal weather patterns. For example, a strikingly diverse *Chenopodium* community consisting of at least seven species (*C. album*, *C. berlandieri*, *C. hians*, *C. incanum*, *C. leptophyllum*, *C. neomexicanum* and/or *C. fremontii*, and *C. watsonii*) and potential hybrids that were thriving along the Verde River bottom near Cottonwood, Arizona, in 2005 was revisited and found to be entirely absent in each of the next two summers (Fig. 3.6). We have observed a similar pattern at sites in Nevada, Utah, eastern Colorado, Wyoming, and California, over the span of years between 2004 and 2008. This implies that goosefoot diversity may be harbored over long time periods within the soil's seed bank. Protecting these seed banks is crucial to maintaining the biodiversity of the genus. At the same time, however, it is extremely difficult to sample and assess wild *Chenopodium* biodiversity because there may be few or no green plants upon which to base the assessment at a given location within a given year, while the next year the site might contain a profusion of goosefoot biodiversity. Surveying and/or collection



Fig. 3.6 Wild *Chenopodium* collection site on sandy soil along the embankment of the Verde River at Cottonwood, Arizona, in July 2005. The following two summers *Chenopodium* was essentially absent from the location. (**a**) Representative biodiversity for leaf and stem morphology at the site. (**b**) Overview of the site

expeditions therefore need to plan carefully based on the weather in the target area over the course of the previous 6 or so months, taking into account factors such as contributions of fall rain and winter snow cover to soil moisture, winter temperatures, date of last frost, growing-degree days, etc.

3.3 *Chenopodium* Genetics

For all the chenopods, the basic chromosome number is $x = 9$. There is some confusion if *Dysphania* species are also included, as a number of these have $x = 8$ (Giusti 1970). Some reported chromosome number counts are listed in Table 3.4. Karyologically, the species are distributed in three different polyploidy levels: diploid, tetraploid, and hexaploid. Diploids have $2n = 2x = 18$ chromosomes (e.g., *C. atrovirens*, *C. californicum*, *C. ficifolium, C. pallidicaule*, etc.). Tetraploids, such as *C. quinoa*, *C. strictum*, and *C. berlandieri*, have $2n = 4x = 36$ chromosomes. The highest ploidy level found thus far is hexaploid, possessing $2n = 6x = 54$ chromosomes, as represented by *C. album* and *C. bushianum*. However, there are curated accessions identified morphologically as *C. album* that differ for their chromosome numbers, including all three polyploidy levels (Tanaka and Tanaka 1980; Wang et al. 1993; Bhargava et al. 2007; Kolano et al. 2008).

3.3.1 Chromosome and Genome Size

Detailed studies of *Chenopodium* cytogenetics have been done for only a few species. Karyotypes of the genus consist mostly of metacentric and submetacentric chromosomes. Some reports describe only one pair of chromosomes with a secondary constriction in diploid and tetraploid species, although Palomino et al. (2008) reported two pairs in *C. quinoa* and *C. berlandieri* ssp. *nuttaliae* and hexaploid *C. album* has been reported to contain two pairs of satellited chromosomes (Kolano et al. 2001, 2008; Bhargava et al. 2006). We have also observed that species belonging to the *C. album* aggregate (subg. *Chenopodium*) exhibit similar karyotypes. Several authors have suggested that karyotype differentiation pattern may correspond to the sectional classification of the genus (Bhargava et al. 2006; Palomino et al. 2008). Within each species, there is minor karyotypic variation; this variation is likely due to chromosome rearrangements (mainly pericentric inversion and translocations), which are being maintained due to the predominantly self-pollinating behavior of most species of the genus (Risi and Galwey 1984; Bhargava et al. 2006; Palomino et al. 2008).

Several studies have examined genome size, albeit for only a few *Chenopodium* and *Dysphania* species;

Table 3.4 Chromosome number and ploidy level of *Chenopodium* species in the literature

Species	Ploidy	Chromosome number	References
C. acuminatum	4*x*	36	Tanaka and Tanaka (1980)
C. album	2*x*, 4*x*, 6*x*	18, 36, 54	Tanaka and Tanaka (1980), Gangopadhyay et al. (2002), Kolano et al. (2006), Bhargava et al. (2007)
C. amaranticolor (*C. giganteum*)	6*x*	54	Bhargava et al. (2005, 2007)
C. atrovirens	2*x*	18	Authors
C. berlandieri	4*x*	36	Wang et al. (1993)
C. bushianum	6*x*	54	Bhargava et al. (2007)
C. californicum	2*x*	18	Authors
C. carnosolum	2*x*	18	Giusti (1970)
C. cordobense	2*x*	18	Giusti (1970)
C. feotidum	2*x*	18	Bhargava et al. (2007)
C. ficifolium	2*x*	18	Bhargava et al. (2007)
C. fremontii	2*x*	18	Authors
C. glaucum	2*x*	18	Wang et al. (1993)
C. hircinum	4*x*	36	Wang et al. (1993)
C. hybridum (*C. simplex*)	2*x*	18	Bhargava et al. (2007)
C. litwinowii	2*x*	18	Uotila (1993)
C. macrospermum	2*x*	18, 36	Authors; Giusti (1970)
C. murale	2*x*	18	Gangopadhyay et al. (2002)
C. neomexicanum	2*x*	18	Wang et al. (1993)
C. obscurum	2*x*	18	Giusti (1970)
C. opulifolium	4*x*	36	Bhargava et al. (2007)
C. pallidicaule	2*x*	18	Giusti (1970) and Bhargava et al. (2007)
C. papulosum	2*x*	18	Giusti (1970)
C. pratericola	2*x*	18	Giusti (1970)
C. quinoa	4*x*	36	Wang et al. (1993)
C. scabricaule	2*x*	18	Giusti (1970)
C. strictum	6*x*	54	Bhargava et al. (2006)
C. suecicum	2*x*	18	Uotila (1978)
C. ugandae	4*x*	36	Bhargava et al. (2007)
C. urbicum	2*x*	18	Kawatani and Ohno (1956)
C. vulvaria	2*x*	18	Kawatani and Ohno (1956)
C. watsonii	2*x*	18	Authors

however, large differences between species were revealed (Table 3.5). Among studied species, the 2C nuclear DNA content showed a 7.9-fold variation ranging from 0.63 to 4.95 pg. The highest interspecies differences in 2C DNA content were observed among diploid species. Among analyzed diploids, the smallest genome was possessed by *C. aristatum* (now *Dysphania aristata*, 0.63 pg) and the largest by diploid *C. album* (1.8 pg). Less variation was observed among tetraploid and hexaploid species, with an approximate genome size between 724 and 967 megabase pairs (Stevens et al. 2006; Bhargava et al. 2007; Kolano et al. 2008; Palomino et al. 2008). Palomino et al. (2008) reported no statistically significant genome size differences in *C. quinoa* and *C. berlandieri* subsp. *nuttalliae* accessions. In contrast, hexaploid *C. album* had clear differences in 2C value between accessions of European origin and those of Indian origin. This may not be surprising, since *C. album* is well known for its tremendous heterogeneity across its worldwide range (Bhargava et al. 2007; Kolano et al. 2008).

3.3.2 Interspecific and Intergeneric Hybridization

There are relatively few published reports of interspecific hybridization in *Chenopodium* that do not involve the *quinoa–berlandieri–hircinum* 4*x* complex. Wilson (1980) made 17 intersubsectional crosses onto male-sterile quinoa and was only able to recover one sterile hybrid, with the South American diploid *C. petiolare*. He was also able to produce sterile

Table 3.5 Genome size and ploidy level of *Chenopodium* and *Dysphania* species

Species	Ploidy	2C DNA (pg)	References
C. album	2*x*	1.44 – 1.80[a]	Bhargava et al. (2007) and Kolano et al. (2008)
C. album	4*x*	3.26 ± 0.04	Bhargava et al. (2007) and Kolano et al. (2008)
C. album	6*x*	3.85 – 4.95[a]	Bhargava et al. (2007) and Kolano et al. (2008)
C. aristatum (*D. aristata*)	2*x*	0.625 ± 0.02	Bhargava et al. (2007)
C. berlandieri subsp. *nuttallie*	4*x*	2.96 – 3.04[a]	Palomino et al. (2008)
C. botrys (*D. botrys*)	2*x*	0.875 ± 0.03	Bhargava et al. (2007)
C. bushianum	6*x*	4.65 ± 0.04	Bhargava et al. (2007)
C. feotidum	2*x*	0.89 ± 0.02	Bhargava et al. (2007)
C. ficifolium	2*x*	1.325 ± 0.01	Bhargava et al. (2007)
C. giganteum	6*x*	4.39 ± 0.04	Bhargava et al. (2007)
C. murale	2*x*	1.245 ± 0.02	Bhargava et al. (2007)
C. opulifolium	4*x*	2.67 ± 0.03	Bhargava et al. (2007)
C. pallidicaule	2*x*	1.26 ± 0.02	Bhargava et al. (2007)
C. quinoa	4*x*	2.01 – 3.24[a]	Stevens et al. (2006), Bhargava et al. (2007) and Palomino et al. (2008)
C. strictum	6*x*	4.46 ± 0.03	Bhargava et al. (2007)
C. ugandae	4*x*	2.86 ± 0.03	Bhargava et al. (2007)

[a]Accessions differed in 2C DNA value

hybrids using pollen from the intrasubsectional diploid *C. neomexicanum* and backcross-capable hybrids with 6*x* *C. bushianum*. Wilson's (1980) quinoa × wild *C. berlandieri* and quinoa × *C. berlandieri* subsp. *nuttaliae* hybrids were also fertile, though in a previous, and more exhaustive, study *C. quinoa* × *C. berlandieri* subsp. *nuttaliae* hybrids had low pollen stainability and were self-sterile (Wilson and Heiser 1979). Interestingly, 4*x* × 6*x* crosses involving *C. berlandieri* and *C. bushianum* had substantially higher percent pollen stainability than the quinoa × *C. bushianum* hybrids: 71–97% versus 1–8% (Wilson 1980).

In a later study, Wilson and Manhart (1993) reported that over 30% of progeny of wild *C. berlandieri* plants free-living around the periphery of highly diverse quinoa introduction fields in eastern Washington State were derived via passive outcrossing from the quinoa plants. Their conclusion was based on the presence of polymorphic quinoa isozyme alleles as well as morphologically intermediate leaves.

3.3.3 Molecular and Genomics Resources

Few molecular studies have been conducted in the chenopods, although increased international interest has prompted more recent studies. Initial molecular studies in quinoa sought to elucidate the genetic relationships between several *Chenopodium* species. Wilson (1988a) used allozyme data to construct a phylogenetic tree of *Chenopodium* species which distinguished coastal and Altiplano ecotypes. The data also supported the hypothesis of the Altiplano being the center of origin for quinoa. Similar studies were conducted using seed protein variation and morphological markers (Wilson 1988b; Fairbanks et al. 1990).

Fairbanks et al. (1993) were the first to use molecular markers in quinoa. They used random amplified polymorphic DNA (RAPD) primers to detect polymorphisms among different quinoa accessions. Bonifacio (1995) also used RAPDs as a means to identify true hybrids from intergeneric crosses. Ruas et al. (1999) used RAPDs to investigate the relationship among 19 *Chenopodium* species, and found that accessions clustered according to their species classifications.

More recent studies have utilized markers other than RAPDs. For example, Mason et al. (2005) developed simple sequence repeat (SSR) or microsatellite markers for use in quinoa by sequencing 1,276 clones from enriched CA, ATT, and ATG repeat DNA libraries. They found that of 397 potential SSRs, 208 were polymorphic on a panel of 31 quinoa accessions. In addition, the markers were tested on three different *Chenopodium* species (*C. pallidicaule*, *C. giganteum*, and *C. berlandieri* ssp. *nuttalliae*). Sixty-seven percent of the SSRs amplified in all species, while 99.5% amplified in *nuttalliae*, illustrating its close relationship with quinoa. Christensen et al. (2007) used 35 of

the these SSR markers to assess the level genetic diversity in 152 accessions (representing the USDA and CIP collections) of quinoa and to test four hypotheses regarding quinoa's center of diversity, Highland and Lowland clustering patterns, origin of lowland varieties and the origin of domestication.

In addition to SSRs, single nucleotide polymorphism (SNP) markers have been developed in quinoa. Coles et al. (2005) obtained and deposited 424 expressed sequence tags (ESTs) in GenBank. Of these ESTs, 349 were found to have homology to protein-encoding genes from other plants. Fifty-one SNPs were identified from 20 of these ESTs, of which 38 were single-nucleotide changes and 13 were insertion/deletion changes. In addition, 81 more SNPs were obtained when quinoa was compared to *C. berlandieri* ssp. *nuttalliae*.

Maughan et al. (2004) constructed the first genetic linkage map of quinoa. The map was based on 80 F_2 individuals from a cross between Ku-2 (a Chilean lowland type) and 0654 (an Altiplano type) and consisted of 230 amplified fragment length polymorphism (AFLP), 19 SSR, and six RAPD markers. The map spanned 1,020 cm and contained 35 linkage groups, with an average marker interval of 4.0 cm. Using the method of Hulbert, the total genome length was predicted to be 1,700 cm. Jarvis et al. (2008) reported the detection of 216 new SSR markers and a new genetic linkage map was constructed for a recombinant inbred line (RIL) population. The new linkage map included the location of two 11S seed storage protein loci, the nucleolar organizing region (NOR) and 275 molecular markers, including 200 SSR markers. The map consists of 38 linkage groups (LGs) covering 913 cm. Segregation distortion was observed in the mapping population for several marker loci, indicating possible chromosomal regions associated with selection or gametophytic lethality.

Four hundred twenty-four polymorphic quinoa SSR markers were evaluated for polymorphism and used to assess the level of genetic diversity in 47 cañahua (*C. pallidicaule*) accessions (Vargas personal communication). Sixty-three polymorphic microsatellite markers were identified, amplifying a total of 306 alleles. The number of alleles per marker identified ranged from 2 to 15 with an average of 4.86 alleles per marker locus. Phylogenetic analysis of the SSR data clearly identified two major groups of cañahua accessions, although the reason for this clustering remains unclear at this time.

Stevens et al. (2006) reported the development of a bacterial artificial chromosome (BAC) library for quinoa. The library, which was estimated to represent approximately ninefold coverage of the haploid quinoa genome, contains 26,880 clones from *Bam*HI digests and 48,000 clones from *Eco*RI digests. The BAC library was recently used as a starting point to clone and characterize two homoeologous *Salt Overly Sensitive* 1 loci (*cqSOS1A* and *cqSOS1B*) from quinoa, including full-length cDNA sequences, genomic sequences, relative expression levels, fluorescent in situ hybridization (FISH), and a phylogenetic analysis of *SOS1* genes from 13 plant taxa (Maughan et al. 2009). The *SOS1* gene encodes a plasma membrane Na+/H+ antiporter that plays an important role in germination and growth of plants in saline environments.

Maughan et al. (2006) sequenced the nucleolar organizing region (NOR), intergenic spacers (IGS) and 5S rDNA non-transcribed spacers (NTS) from five quinoa and one *C. berlandieri* accession. IGS sequences revealed length differences due to insertion/deletions (indels), differing numbers of repeat copies, and other rearrangements among the accessions. NTS sequencing revealed two sequence classes, likely representing one locus from each of the genomes in allotetraploid quinoa.

3.4 Potential Economic Value

Although the nutritional and industrial value of quinoa has been highly touted in the scientific and popular literature, relatively little research has been done on the nutritional value, biotic or abiotic stress resistances, or other potentially valuable characteristics of its wild relatives. Much of the research on nutritional quality of wild species originated in India and was focused on *C. album* and other wild species native to Eurasia. Dembitsky et al. (2008) identified a wide range of potentially valuable volatile metabolites and active monoterpenoids in 11 *Chenopodium* species, including quinoa. Guil et al. (1997) reported that *C. album* seeds are an exceedingly rich source of ascorbic acid.

One of the most comprehensive early studies of wild *Chenopodium* nutritional value was by Prakash et al. (1993). They measured the nutritional components – protein, carotenoids, and vitamin C – in the leaves of 11 species or interspecies hybrids: *C. album*,

C. album × *quinoa*, *C.* (*Dysphania*) *ambrosioides*, *C. amaranticolor* (*giganteum*), *C. berlandieri*, *C. foliosum* × *polyspermum*, *C. murale*, *C. opulifolium*, *C. quinoa*, *C. striatum*, and *C. foliosum* × *polyspermum* × *quinoa*. Prakash et al. (1993) also measured seed protein, fat, and fatty acid composition in eight of these accessions. They detected quantities of seed protein ranging from 142 g/kg in *C. opulifolium* to 106 g/kg in *C. quinoa* and fat content up to 62 g/kg (in *C. murale*). Pale-seeded accessions had lower seed contents than did black-seeded ones. Leaf ascorbic acid values were as high as 2.4 g/kg fresh weight (in *C. berlandieri*) and carotenoids ranged from a low of 78 mg/kg (in *C. album*) up to 190 mg/kg fresh weight (in *C. quinoa*). However, they also detected up to 5.0 g/kg nitrate (in *C. murale*) and 39 g/kg oxalates (in *C. ambrosioides*) in the leaves.

A recent study by Bhargava et al. (2008) quantified ten minerals in leaves of 40 *Chenopodium* accessions comprising seven species (*C. album*, *C. berlandieri* subsp. *nuttaliae*, *C. bushianum*, *C. giganteum*, *C. murale*, *C. quinoa*, and *C. ugandae*). They reported very high potassium (2–8.5 g/100 g dry weight), calcium (generally 1.0–1.2 g/100 g dry weight), iron, and magnesium contents, along with sodium. In addition, they identified genotypes of quinoa that hyper-accumulate heavy metals, a potentially valuable characteristic for bioremediation of heavy metal-contaminated soils.

In addition to these studies, extensive anecdotal evidence supports the enormous potential of just *C. berlandieri*, let alone other less common wild species, as potential sources of resistance genes for improving cultivated quinoa. For example, in a field planting of some CIP-FAO quinoa nursery lines at Rexburg, Idaho, in 2003, while the quinoa plants acquired a severe infestation of leaf miners, pitseed goosefoot plants growing between the poorly tended rows showed essentially no damage from the insects. Similar observations have been made in PROINPA fields in Bolivia infested with downy mildew (Bonifacio and Vargas unpublished).

3.5 Conclusions

Considering the potential utilization of wild genetic resources in *Chenopodium*, the two greatest problems have to be addressed and they are (1) the incomplete taxonomic classification of the genus and (2) the meager amount of wild material readily available in germplasm collections. Obviously, the two problems are intricately interrelated. With an ever-increasing toolbox of molecular and genomic resources from quinoa research, the taxonomic classification issue should begin to be resolved, at least for major species that are readily accessible, in the near future. For example, Maughan et al. (2009) and Soliai et al. (2009) reported the identification of locus-specific, orthologous DNA sequence variants for introns 16 and 17 of the *SOS1* gene in quinoa, wild *C. berlandieri*, *C. bushianum*, and *C. hircinum*, which are in the process of being matched to sequences from a series of diploid species from Eurasia along with New World species collected since 2004. In addition, Soliai et al. (2009) provided a preliminary report on a phylogenetically informative array of sequenced 5S ribosomal RNA (rRNA) gene non-transcribed spacer (NTS) clones from over 25 mostly New World species. Molecular markers and sequence information are also revealing their power to ensure that *Chenopodium* gene banks can devote limited resources to maintain maximally diverse accessions.

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Chapter 4
Cicer

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4.1 Introduction

Chickpea (*Cicer arietinum* L.) is one of the earliest grain crops cultivated by man and has been found in Middle Eastern archeological sites dated at 7500–6800 BC (Zohary and Hopf 2000). Chickpea is grown in about 50 countries with an estimated 95% of the cultivated area in the developing countries. Chickpea production is particularly important in the countries of South Asia and accounts for about 71% of global area devoted to the crop. Chickpea can fix up to 140 kg nitrogen ha^{-1} and meet up to 80% of its nitrogen requirement from symbiotic nitrogen fixation (Saraf et al. 1998). Substantial amounts of nitrogen remain in the soil following the chickpea crop, which is beneficial to subsequent crops. Chickpea crop residues add much needed organic matter for the maintenance of soil health, long-term fertility, and sustainability of the ecosystems. Chickpea is an important source of protein for millions of people in developing countries. Chickpea has the highest nutritional compositions of any dry edible grain legume and does not contain any anti-nutritional factors. In addition to having high protein content (20–22%), chickpea is rich in fiber and minerals (phosphorus, calcium, magnesium, iron, and zinc), and its lipid fraction is high in unsaturated fatty acids (Williams and Singh 1987). Chickpea contains higher amounts of carotenoids such as β-carotene than genetically engineered "golden rice" (Abbo et al. 2005).

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4.2 Origin and Taxonomy

Chickpea is a Neolithic crop that had its origin in the fertile crescent some 10,000 years ago (Lev-Yadun et al. 2000; Zohary and Hopf 2000). Evidence suggests the region of southeastern Turkey and adjoining Syria (van der Maesen1987) as the center of origin. The progenitor species of chickpea, *Cicer reticulatum*, grows there even today. Further evidence of its origin comes from seeds that date back to 5450 BC that have been unearthed from excavations at Hac near Burdur in Turkey (Helbaek 1970). From Turkey, chickpea diverged in two directions: one into the Western Province, where it is grown in spring and summer, and the other into the eastern and southern parts of the region, where it is grown in the cool dry season.

Various studies have shown that chickpea rests on a narrow genetic base (Millan et al. 2006) and this is the reason for the crop being susceptible to a range of diseases and pests. According to Abbo et al. (2003), a series of four evolutionary bottlenecks are responsible for the narrow genetic base of the crop. Unlike pea, barley, lentil, and wheat, the progenitor species of chickpea, *C. reticulatum* (Ladizinsky and Adler 1976), is narrowly distributed in southeastern Turkey and harbors limited adaptive variation compared to those of wheat or barley (Berger et al. 2003). Based on both genetic and archeological evidence (Zohary 1999; Lev-Yadun et al. 2000), the limited founding species is called the domestic bottleneck. Chickpea is considered to be the victim of a series of bottlenecks. The crop underwent a bottleneck when it was shifted from being an autumn to a spring-sown crop, probably to avoid Ascochyta blight that is less severe in spring-sown crops. Spring-sown chickpea had to be vernalization insensitive, thus further narrowing the genetic

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base. Selection to suit post-rainy season, cropping further reduced the genetic diversity. Replacement of landraces by elite cultivars produced by modern breeding caused yet another bottleneck. With the change in climatic conditions and the evolution of several pathogens, selection will be rigorous for chickpea germplasm to withstand abiotic and biotic stresses. This process of selection will further narrow the genetic base and contribute an additional bottle neck to the chickpea crop.

4.3 Gene Pools of Chickpea

Chickpea is endowed with rich germplasm in the form of wild species. The genus *Cicer* is classified into three gene pools based on its crossability with cultigen. Based on their crossability with cultivated species, wild species, both annual and perennial, have been grouped. Using the classification proposed by Harlan and de Wet (1971), a modification of the classification is proposed. Although the modification does not deviate much from the previously proposed gene pools for chickpea, the secondary gene pool is strengthened by the placement of *C. reticulatum*. The proposed classification is similar to the recent classification proposed by van der Maesen et al. (2007). The primary gene pool consists of cultivated species and landraces. The secondary gene pool consists of the progenitor species, *C. reticulatum* and *C. echinospermum*, a species that is crossable with *C. arietinum* but with reduced fertility of the resulting hybrids and progenies; nevertheless, both are cross-compatible with the cultigen and do not need in vitro interventions to produce hybrids. The tertiary gene pool consists of all the annual and perennial *Cicer* that are not crossable with cultivated *C. arietinum*. All the perennial *Cicer* species are considered to be in the tertiary gene pool as none of the species of this group are known to cross readily with the cultivated species and produce mature seeds (Mallikarjuna and Muehlbauer unpublished results). In general, the transfer of desirable traits from wild species may accompany tightly linked undesirable genes/traits commonly referred to as "linkage drag." Utilization of molecular markers can be used effectively for transfer of genes of interest from wild to cultivated species.

4.3.1 Primary Gene Pool

Cultivated chickpea has vast collections of landraces and improved cultivars that are maintained at the international centers, ICRISAT and ICARDA, and at numerous national gene repositories including USDA, USA. ICRISAT maintains 17,258 accessions (135 wild, 17,123 cultivated). Although a large number of cultivated accessions are conserved in the gene bank, there is fatigue in the utilization of the germplasm, as the sheer numbers are intimidating. ICRISAT chickpea breeders used just 83 germplasm lines, in contrast to their use of 480 breeding lines for the development of 3,430 advanced varieties (ICCV numbers) during the same period (1978–2004) (Upadhyaya et al. 2008). India has released 126 cultivars between 1967 and 2003. Pedigree analysis of 86 cultivars developed from crosses has revealed that although 95 progenitors were involved, just ten of these contributed 35% of the genetic base (Shivkumar et al. 2004). The five most frequently used ancestors were Pb7, IP 58, F 8, Rabat, and S 26. Furthermore, about 41% of the cultivars developed through crossing have Pb7 as an ancestor. This suggests that many cultivars share a narrow genetic base. Further, a global composite collection was developed that comprised 3,000 accessions. The composite collection is made up of 80% landraces, 9% advanced breeding lines, 2% cultivars, 1% wild species, and 8% whose precise status is unknown (Upadhyaya et al. 2008). It is envisaged that such collections would enhance the utilization of germplasm in improvement of chickpea. This is an ideal set of germplasm for allele mining, association genetics, mapping and cloning gene(s), and applied breeding. Nevertheless, due to narrow ancestry and numerous bottlenecks, chickpea has limited genetic variation in the primary gene pool. In spite of the above-mentioned constraints, extensive international breeding efforts have led to the development of over 300 improved varieties (Gowda and Gaur 2004). Potential yield of chickpea is estimated to be 5.0 ton ha^{-1}, but the average yield is around 0.8 ton ha^{-1} as various biotic and abiotic stresses reduce yield. Drought is a serious abiotic stress, which has led to the development of cultivars that can escape terminal drought through early maturity. ICRISAT has invested in the development of short-duration cultivars such as ICCV 2, ICCV 3, and KAK 2.

Chickpea is highly self-pollinating with an outcrossing rate of less than 1%. Two main types of chickpea cultivars are grown globally, representing two diverse subgene pools: Kabuli and Desi. The Kabuli types are generally grown in the Mediterranean region, southern Europe, western Asia, and northern Africa and the Desi types are grown mainly in Ethiopia and the Indian subcontinent.

4.3.2 Secondary Gene Pool

C. reticulatum and *C. echinospermum* are the two wild species in the secondary gene pool. *C. reticulatum* crosses with *C. arietinum,* resulting in completely fertile hybrids and progenies. *C. reticulatum* is considered as the wild progenitor of *C. arietinum* based on morphological and cytological similarities as well as crossability (Ladizinsky and Adler 1976), molecular analysis, and their cohabitation at the center of origin of the crop (Zohary and Hopf 1988). Many accessions of *C. reticulatum* have been identified with useful genetic variation and there are various reports (see below) on its utilization to introgress useful traits into the cultivated species (Ascochyta blight resistance: Collard et al. 2003; botrytis gray mold resistance: Mallikarjuna unpublished data; *Helicoverpa armigera* resistance: Mallikarjuna et al. 2007a; nematode resistance: Malhotra et al. 2002; high yield: Singh and Ocampo 1997). Hence, *C. reticulatum* represents a potential source of genes for broadening the genetic base of cultivated chickpea.

Another potential source of increased genetic variation is *C. echinospermum*. Natural hybrids between chickpea and *C. reticulatum* and between *C. echinospermum* and *C. reticulatum* have been reported by ICARDA (Irula et al. 2002), which strongly suggests compatibility among these three species and the reason for compatible pollinations. Mature seed set is observed, when these species are crossed with each other. Although *C. echinospermum* readily crosses with cultivated chickpea, cytological studies have shown meiotic abnormalities leading to certain degrees of pollen sterility (Pundir and Mengesha 1995). Molecular studies have shown that *C. echinospermum* is not as closely related to cultivated chickpea as *C. reticulatum* (Choumane et al. 2000).

Ladizinsky and Adler (1976) reported that *C. reticulatum* and *C. echinospermum* differed from each other by a major reciprocal translocation, and their hybrid was completely sterile. They also stated that *C. echinospermum* also differed from the cultigen by the same translocation and their hybrids were also sterile. However, with some accessions of *C. echinospermum*, it was possible to obtain completely fertile selections (Singh and Ocampo 1997). Collard et al. (2003) used an accession of *C. echinopsermum* (PI 527930) and developed an interspecific population and identified quantitative trait loci (QTL) associated with seedling resistance to Ascochyta blight. The same accession of *C. echinospermum* crossed with a different cultivar of chickpea set mature seeds, but the F_1 hybrid did not have fertile pollen grains. Meiosis was normal until the tetrad stage after which, instead of tetrads, polyads were observed. It was not possible to obtain F_2 seeds, but backcrossing the F_1 hybrid with female parent, chickpea cultivar KAK 2 gave rise to mature seeds (Mallikarjuna unpublished results). This shows that genotype of the female parent is important while attempting crosses with wild *Cicer* species and that crosses with *C. echinospermum* often have disturbed meiosis (Pundir and Mengesha 1995). The rationale of placing both the species in the secondary gene pool is their crossability with the cultigen.

4.3.3 Tertiary Gene Pool Species

Annual *Cicer*: Many of the species in this group harbor important traits/genes necessary for the improvement of chickpea, such as *H. armigera* resistance in *C. judaicum*, *C. pinnatifidum*, and *C. bijugum* (Sharma et al. 2005a); Ascochyta blight resistance in *C. judaicum*, *C. bijugum*, and *C. pinnatifidum* (Pande et al. 2006); botrytis gray mold resistance in *C. judaicum* (Pande et al. 2006), and drought resistance in *C. pinnatifidum* (Bhattarai and Fettig 2005). Various studies have shown that wild species in the tertiary gene pool are distantly related to cultivated chickpea; however, crossing techniques are being improvized for making wide crosses in chickpea using tertiary gene pool species (Mallikarjuna 1999; Mallikarjuna et al. 2007c; Mallikarjuna and Jadhav 2008). Many of the incompatible *Cicer* species in the tertiary gene pool that do not cross easily with cultivated chickpea have been used in crosses followed by hormone applications, embryo rescue, and attempts to root hybrid shoots/plants in vitro. Although hybrid

shoots/plants have been obtained, hybrids have proven to be fragile and have not withstood transfer to glass-house/field and hence are not yet available for chickpea improvement.

4.3.3.1 Barriers to Interspecific Crosses

It is now known that the barriers to hybridization are post-zygotic (Ahmad et al. 1988; Mallikarjuna 1999) meaning that pollinations take place but the zygote, which is a few celled, begins to abort by 3–5 days after pollination. Badami et al. (1997) were able to postpone the abscission of pollinated pistils to 15–18 days by the application of growth regulators. This facilitated the growth of the hybrid embryo to early cotyledonary stage of development and being 0.5–1.0 mm in size (Mallikarjuna 1999).

Embryos of size 0.5 mm or less did not grow directly on culture medium while 0.3–0.4 mm size embryos responded well to specific growth hormones when cultured as in-ovulo embryo culture. Embryo response was maximum when Zeatin was used in combination with indole acetic acid in in-ovulo embryo culture medium. Hybrid embryos emerged out of the ovules after 3–4 weeks of culture (Mallikarjuna 1999). Similar response was not obtained when zeatin was replaced with other cytokinins, which reduced the number of responding embryos (Mallikarjuna unpublished). In peanut ovule culture, emergence of the developing embryo is observed as seen in chickpea (Mallikarjuna and Sastri 1985), but in pigeonpea ovule culture, the developing embryo never emerges out of the ovule; hence, in such cases, it is important to dissect the developing embryo out of the ovule.

The best time to save the aborting seeds/ovules was when the hybrid embryo had reached its maximum growth and development, being at the cotyledonary stage of development, which was 15–18 days after pollination. If left longer on the plant, the pods turn yellow, indicating abortion of the hybrid seed. It was possible to save aborting hybrid embryos from the cross *C. arietinum* × *C. pinnatifidum* by in-ovulo embryo culture. Some of the hybrid shoots were pale yellow in color and scanning electron microscopy (SEM) studies showed that the chloroplasts were abnormal. Use of a cytokinin in culture medium in combination with light helped the conversion of leucoplastids to chloroplasts (Badami et al. 1997). Overall, the hybrids between *C. arietinum* and *C. pinnatifidum* were fragile with the leaves resembling those of *C. pinnatifidum*. The color of the flower was pale violet resembling the violet color of the male parent and the pollen was 100% non-viable (Mallikarjuna 1999).

In an attempt to check if *C. reticulatum* and *C. echinospermum* could be considered bridge species, as a means of transferring of genes from species of the tertiary gene pool to the cultigen, crosses were carried out between *C. reticulatum* and *C. pinnatifidum* and between *C. echinopsermum* and *C. pinnatifidum*. In both the crosses, embryos from crossings aborted 15–20 days after pollination. Rescuing hybrid embryos in vitro gave rise to albino plants (Mallikarjuna and Jadhav 2008). Although mature seeds were not obtained in the cross between *C. reticulatum* and *C. bijugum*, hybrid shoots were green (Mallikarjuna et al. 2007a). It is suggested that a larger number of *C. reticulatum* accessions should be used to identify compatible combinations to obtain viable and green hybrid plants between *C. reticulatum*/*C. echinospermum* and *C. pinnatifidum*.

Crosses between incompatible annual *Cicer* species as the female parent (*C. pinnatifidum* and *C. bijugum*) and cultivated chickpea as the pollen donor produced mature seeds consistently. These presumed hybrid seeds germinated well and all the plants resembled the female parent. Molecular profiling and high pollen fertility of the presumed hybrids indicated that the plants were identical to the female parent. In the crosses with cultivated chickpea and *C. cuneatum*, a similar situation was observed (Gaur personal communication). Although further investigations are necessary, the results may indicate apomictic seed production. This type of seed production is most prevalent when *C. pinnatifidum* or *C. bijugum* were used in crosses with cultivated chickpea as the pollen donor (Mallikarjuna 2003).

Development of haploid plants from chickpea anther culture and microspore culture is now possible (Grewal et al. 2009). Mallikarjuna et al. (2005) demonstrated that multicellular microspores can also be obtained through wide cross between chickpea and *C. pinnatifidum*. The hybrid gave rise to multicellular microspores in large numbers with divisions in all the microspores in some plants. Culturing such microspores may give rise to haploid plants that could be

further utilized, through chromosome doubling, for gene transfer to *C. arietinum*.

4.3.3.2 Perennial *Cicer*

There are 34 perennial wild *Cicer* species, which require very specific soil and environmental conditions for growth and reproduction. Traits of interest such as resistance to Ascochyta blight (Muehlbauer et al. 1994), *H. armigera* (Sharma et al. 2006), Fusarium wilt (Kaiser et al. 1994), and drought resistance (Toker et al. 2007) are present in this gene pool. Perennial *Cicer* species survived the severe frost conditions and resumed their vegetative growth with the onset of summer in the USDA-ARS nursery located at the Washington State University, Pullman, USA. All the perennial *Cicer* species have larger plant morphology compared to the annual *Cicer* species, with robust vegetative growth (Fig. 4.1a, b). The flowers are larger and produce multi-seeded fruits/pods (Fig. 4.1c, d). Some of the perennial *Cicer* species have morphological modifications, such as spines and tendrils. Some of the characters that chickpea would benefit from perennial *Cicer* are large and robust vegetative growth, large pods with multiple seeds (Fig. 4.1c, d), drought and cold tolerance, Ascochyta blight, and insect resistance.

Application of growth regulators was mandatory to obtain pod set in the crosses involving perennial *Cicer* (Table 4.1). Pollinations were growth regulator (gibberellic acid 75 mg/L + naphthalene acetic acid 10 mg/L + kinetine 10 mg/L)-aided. Without the application of growth regulators, immature pods aborted by 6 days after pollination. Growth regulators were able to retain the pods from cross pollinations for 15 days or more. Pollinated pistils were observed under a microscope for pollen response. Pollen grains germinated on the stigma (Fig. 4.1e) and post-fertilization changes were observed (Fig. 4.1g, h).

Microscopic techniques were used to determine the barriers to successful hybridization between *C. arietinum* and *C. anatolicum*. Fluorescence microscopy of pollen tube growth and development in these hybrid crosses showed that pollen was able to germinate, and the resulting pollen tubes penetrated the stigma, style, ovary, and ovule tissues. Traditional light microscopy was used to examine *C. arietinum* and *C. anatolicum* hybrid embryo and endosperm development. When *C. arietinum* (cultivar Myles) and *C. anatolicum* (PI 561078) were self-fertilized resulting in the formation of a zygote and endosperm nucleus (Figs. 4.2 and 4.3, respectively). The zygote developed into an embryo that passed through the globular and heart stages and developed distinct cotyledons by 10 days after pollination. The endosperm nucleus gave rise to the rapidly dividing endosperm tissue. In *C. arietinum* × *C. anatolicum* hybrids, embryo and endosperm growth was arrested after several cell divisions around 4–5 days after pollination and the embryo and endosperm subsequently broke down 6–8 days after pollination (Fig. 4.4). Because the endosperm was non-viable, nutrients available for its development were instead used by the nucellus, which became overgrown. In *C. anatolicum* × *C. arietinum* hybrids, fertilization occurred, but the resulting zygote and endosperm nucleus failed to begin cell division to produce an embryo and endosperm (Fig. 4.5). The post-zygotic failure of this cross is most likely due to a lack of cooperation between the diverse genomes or slow growth of the pollen tube to deliver the gametes before the abscission of the flower, to form and maintain a viable embryo and endosperm.

Crosses were carried out between chickpea cultivar Myles and perennial *Cicer* species *C. oxyodon*, *C. songaricum,* and *C. microphyllum*. Pod set was observed in all the three cross combinations (Fig. 4.1g, h); many of the pods had immature seeds, but none of them matured. Pod set between *C. reticulatum* and *C. oxyodon* was 15%. In other cross combinations using *C. anatolicum*, *C. nuristanicum*, *C. multijugum*, and *C. microphyllum*, pods were observed, but if seeds developed, they were immature and non-viable (Fig. 4.1k). Crosses with *C. echinospermum* and *C. oxyodon* yielded 1% pod set and crosses with *C. songaricum*, *C. microphyllum*, *C. microcanthum*, *C. nuristanicum*, and *C. multijugum* did not set any pods. Crosses between *C. pinnatifudum* and *C. oxyodon* had 10% pod set, and there was no pod set when crosses were made with *C. microcanthum*. Pod set was not observed when *C. judaicum* was crossed with *C. nuristanicum*, *C. macrocanthum*, and *C. oxyodon*. Crosses with *C. bijugum* and *C. oxyodon* yielded 15% pod set, and 30% pod set when crossed with *C. anatolicum*. It was observed that *C. bijugum* was a better female parent with respect to pod set when crossed with perennial *Cicer* species (Table 4.1). But this may not be significant as *C. bijugum* cannot be used as bridge species between



Fig. 4.1 Interspecific hybridization using perennial *Cicer* species. (**a**) *Cicer multijugum* plant. (**b**) Annual *Cicer*. (**c**) *Cicer microphyllum* pod with multiple seeds. (**d**) *C. oxyodon* pod. (**e**) *C. oxyodon* pollinated with *C. reticulatum* pollen. (**f**) *C. multijugum* with *C. oxyodon* pollen. (**g**) Cultivated chickpea with *C. songaricum* pollen. (**h**) Cultivated chickpea with *C. oxyodon* pollen. (**i**) *C. reticulatum* with *C. microphyllum* pollen. (**j**) Perennial *Cicer* with *C. reticulatum* pollen. (**k**) Immature seed from the cross *C. bijugum* × *C. oxyodon*

cultivated chickpea and perennial *Cicer* species as it does not set mature seeds when crossed with cultivated chickpea. Reciprocal crosses using *C. oxyodon* as the female parent and *C. reticulatum* as the pollen donor set a large number of pods (24%; Fig. 4.1j). *C. nuristanicum* had 9% pod set when crossed with *C. reticulatum*. It was possible to retain pods from crossing with perennial *Cicer* species for 15–17 days by application of growth regulators, thereby encouraging the growth of hybrid seeds. But it was not possible to rescue hybrid embryos by culturing them in vitro (Fig. 4.1k).

Hybrid seed size was not more than 2.5–3.0 mm, except for the immature seed from the cross *C. bijugum* × *C. oxyodon* (Fig. 4.1k). Such seeds were collected for in-ovulo embryo culture. Immature seeds showed initial swelling, but no further response even after 60 days of culture. Ovule culture techniques were similar to that used for incompatible crosses involving

annual *Cicer* species (Mallikarjuna 1999). Ovules remained green in culture and enlarged. None of the embryos grew to form a seedling.

4.4 International Transfer of Germplasm

Thus far none of the perennial *Cicer* species have been grown successfully in tropical or subtropical environments where the annual *Cicer* species grow. This is a major impediment for the growth and utilization of perennial species. If the pollen of the perennial *Cicer* species can be preserved for utilization in the regions of the world where these species do not grow, the bottleneck in the utilization of these species to develop crossability techniques can be overcome. This difficulty in the use of perennial *Cicer* germplasm might be overcome through transshipment of viable pollen. Pollen from cultivated and wild species can be collected and preserved for 14 days, at 4–6°C in a desiccator, beyond which the viability of the pollen decreases. Pollinations are successful if the pollen is utilized within 14 days of collection and preservation (Mallikarjuna et al. 2007b).

Table 4.1 Pod set in the crosses between annual and perennial *Cicer* species[a]

Female parent	Male parent	Pod set (%)
Chickpea cv Myles	*C. oxyodon*	18.0
Annual *Cicer*	Perennial *Cicer*	
C. reticulatum	*C. oxyodon*	15.00
C. echinospermum	*C. oxyodon*	1.00
C. pinnatifidum	*C. oxyodon*	10.00
C. bijugum	*C. oxyodon*	15.00
C. bijugum	*C. anatolicum*	30.00
C. bijugum	*C. nuristanicum*	20.00
C. bijugum	*C. Microphyllum*	5.00
Perennial *Cicer*	Annual *Cicer*	
C. microphyllum	*C. reticulatum*	35.0
C. oxyodon	*C. reticulatum*	24.0
C. microphyllum	*C. echinopsermum*	22.00

[a]Crosses were carried out in 2006 at WSU, USA

4.5 Genetic Diversity in *Cicer* Species

A genetic diversity analysis among cultivated and wild chickpea is important for detecting variation in traits of interest. Various biochemical markers can be used; however, only molecular markers are discussed in this review. Narrow ancestry, recent domestication, and high percentage of self-pollination are reflected in the apparent minimal amount of molecular polymorphism between chickpea cultivars (van Rheenen 1992; Udupa et al. 1993). Several molecular markers have detected minimal polymorphism. For example, only 29% of random amplified polymorphic DNA (RAPD) markers were polymorphic and identified narrow genetic distance of 0.09–0.27 within 29 Indian cultivars. Compared to RAPD markers, restriction fragment length polymorphisms (RFLPs) and microsatellite-based markers, such as sequence tagged microsatellite site (STMS) and intersimple sequence repeat (ISSR), detected greater polymorphism in several studies (Serret et al. 1997a, b; Sant et al. 1999;

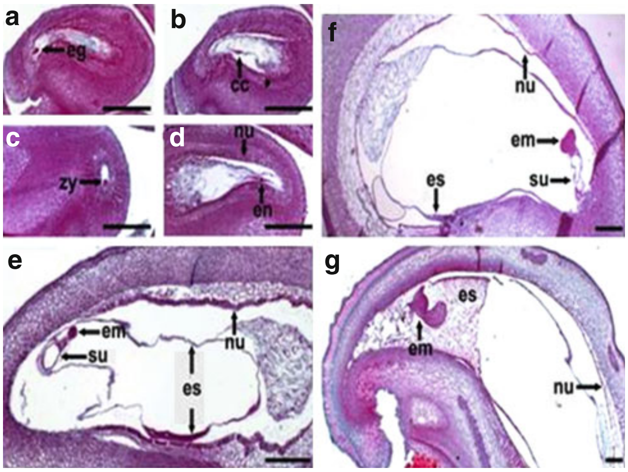


Fig. 4.2 Embryo and endosperm development in self-pollinated *C. arietinum* ovules (**a**, **b**) 0 days after pollination (DAP), (**c**, **d**) 2 DAP, (**e**) 7 DAP, (**f**) 9 DAP, and (**g**) 10 DAP. Scale bars represent 200 µm. *cc* central cell, *eg* egg, *em* embryo, *en* endosperm nucleus, *es* endosperm, *nu* nucellus, *su* suspensor, *zy* zygote

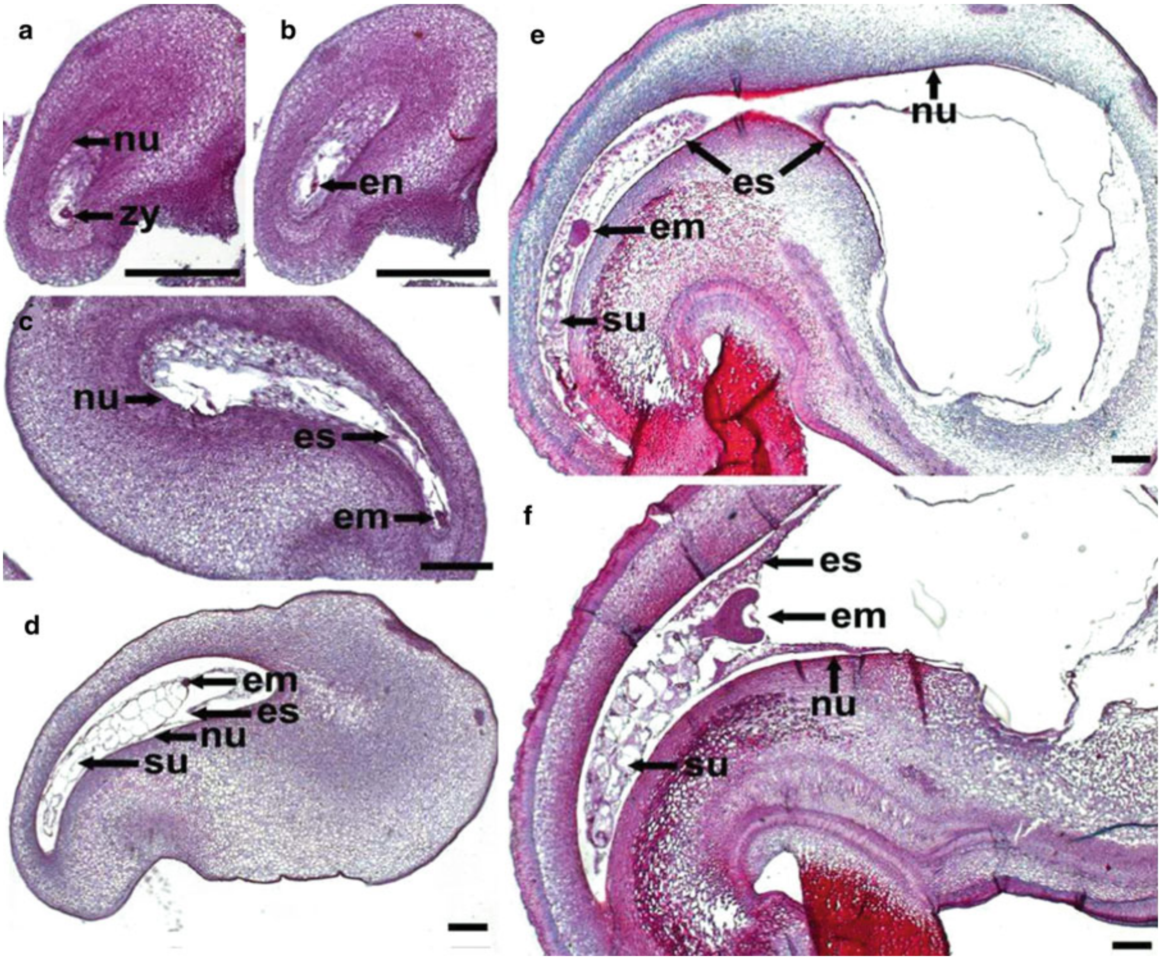


Fig. 4.3 Embryo and endosperm development in self-pollinated *C. anatolicum* ovules (**a**, **b**) 1 day after pollination (DAP), (**c**) 4 DAP, (**d**) 6 DAP, (**e**) 8 DAP, and (**f**) 10 DAP. Scale bars represent 200 µm. *em* embryo, *en* endosperm nucleus, *es* endosperm, *nu* nucellus, *su* suspensor, *zy* zygote

Choumane et al. 2000; Rajesh et al. 2003; Sethy et al. 2006). When oligonucleotide probes were employed, the genetic distance ranged from 0.42 to 0.61 (Sant et al. 1999). Oligonucleotide markers revealed intra- and interaccessional polymorphism in chickpea. Wide range of variability in accessions from Pakistan, Iraq, Afghanistan, Russia, Turkey, and Lebanon were detected. Accessions from India, Jordan, Palestine, Syria, and Iran showed low levels of polymorphism (Sharma et al. 1995; Serret et al. 1997a, b). Sethy et al. (2006) used simple sequence repeat (SSR) markers developed from *C. reticulatum* to study diversity between nine annual *Cicer* species. The study showed greater similarity between cultivated chickpea and *C. reticulatum*. *C. pinnatifidum* was closer to *C. bijugum*, but the two species *C. yamashitae* and *C. chorassanicum* were distinct from all the other species.

Choumane et al. (2000) used STMS markers to study the relationship between the *Cicer* species. Their study showed a close relationship between *C. arietinum*, *C. reticulatum*, *C. echinospermm*, and a perennial *Cicer* species *C. anatolicum*. SSR markers (Staginnus et al. 1999) showed a close relationship between *C. anatolicum* and annual wild *Cicer* species, opening up avenues to consider *C. anatolicum* as the progenitor species of the annual wild *Cicer* species. Using ISSR markers, Rajesh et al. (2002) concluded that annuals are more recent than perennials and might have evolved from the perennial *Cicer* species. Many of the perennial *Cicer* species showed a closer relationship

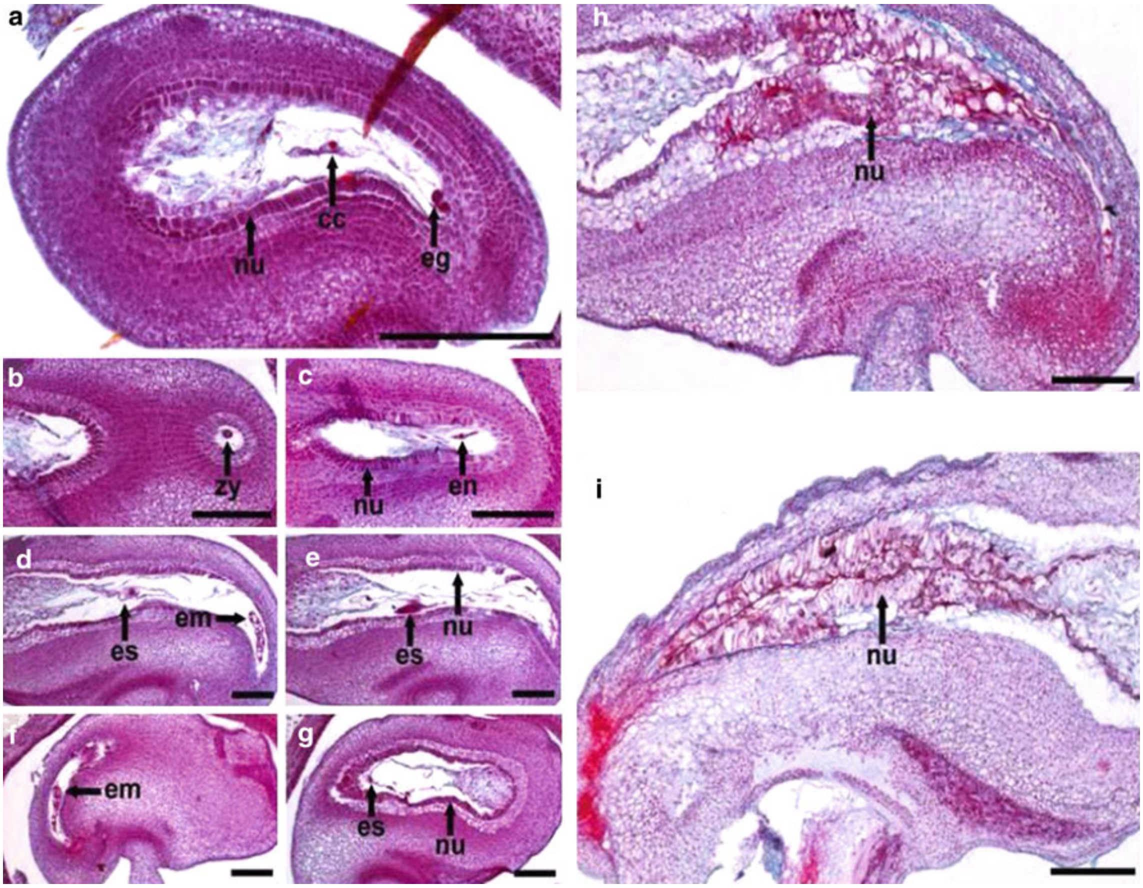


Fig. 4.4 Embryo and endosperm development resulting from the interspecific cross *C. arietinum* × *C. anatolicum* (**a**) 0 days after pollination (DAP), (**b**, **c**) 2 DAP, (**d**, **e**) 4 DAP, (**f**, **g**) 5 DAP, (**h**) 8 DAP, and (**i**) 10 DAP. Scale bars represent 200 μm. *cc* central cell, *eg* egg, *em* embryo, *en* endosperm nucleus, *es* endosperm, *nu* nucellus, *zy* zygote

with cultivated chickpea, *C. reticulatum*, and *C. echinospermum* than with other annual wild *Cicer* species. Here, *C. anatolicum* was more closely related to other perennial *Cicer* species, such as *C. oxyodon* and *C. microphyllum* than to cultivated chickpea or to *C. reticulatum*.

In conclusion, a more comprehensive analysis including a greater number of accessions and a more effective molecular marker system is required to establish a clearer understanding of the phylogenetic relationships among annual and perennial *Cicer* species. It is clear from crossability experiments that *C. reticulatum* is more closely related to cultivated chickpea than any other annual or perennial *Cicer* species. Crossability experiments do not show close relationship between cultivated chickpea and *C. anatolicum*.

4.6 Broadening the Genetic Base by Introducing Useful Genetic Variation from Wild *Cicer* Species

Chickpea is prone to be susceptible to 47 diseases (Nene and Reddy 1987) and 54 insect pests (Reed et al. 1987) attack chickpea. Of these, Fusarium wilt (*Fusarium oxysporum f.* sp. *ciceri*), Ascochyta blight (*Ascochyta rabiei*), root rot (*Rhizoctonia bataticola*), botrytis gray mold (*Botrytis cinerea*), and nematodes (*Meloidogyne incognita*, *M. javanica*, *Heterodera ciceri*) are considered to be globally important. Among the insects, pod borer (*H. armigera*) is the most important pest. Among abiotic stresses, drought

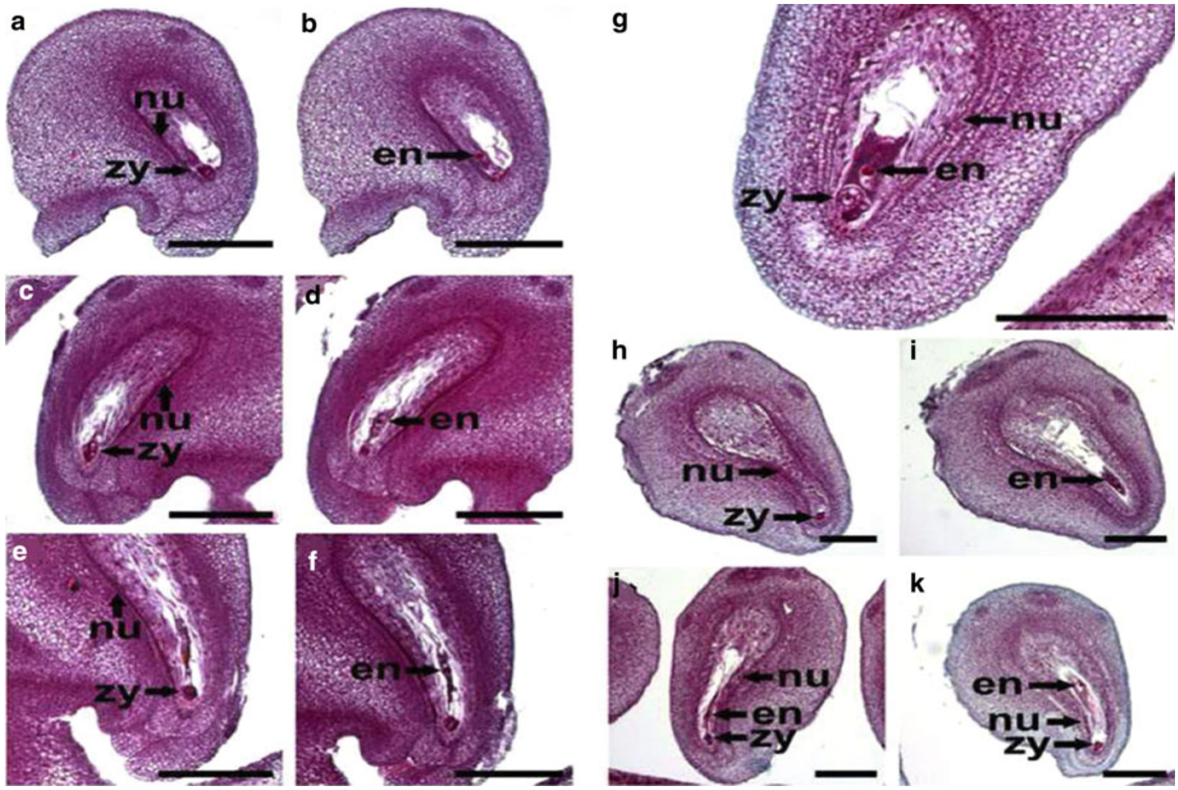


Fig. 4.5 Embryo and endosperm development resulting from the interspecific cross *C. anatolicum* × *C. arietinum* (**a**, **b**) 1 day after pollination (DAP), (**c**, **d**) 3 DAP, (**e**, **f**) 4 DAP, (**g**) 5 DAP, (**h**, **i**) 6 DAP, (**j**) 8 DAP, and (**k**) 10 DAP. Scale bars represent 200 μm. *en* endosperm nucleus, *nu* nucellus, *zy* zygote

is very important. Because of these constraints, world mean yield of chickpea is about 0.8 ton ha^{-1}, which is a serious reduction of yield from an estimated potential of 5 ton ha^{-1}. However, this high estimate is made under very favorable growing conditions not often available for chickpea production. Grain yield is limited by several biotic and abiotic stresses including diseases, such as Fusarium wilt and Ascochyta blight, and abiotic stresses, such as heat and drought. Unavailability of adequate resistance sources to important stresses within the crop species and narrow genetic base are the limitations to its productivity (van Rheenen 1991). Hence, wild relatives with broader diversity can be utilized in breeding chickpea for disease and pest resistance (Lenne and Wood 1991). The resistance to diseases and pests available in germplasm of the primary gene pool is often minimal with a limited number of sources. Nevertheless, resistance to a number of diseases has been incorporated into the elite genotypes. Selection pressure on pathogen populations due to the widespread use of homogeneous host plant resistance may result in more virulent strains, which may overcome the resistance. Hence, discovery and use of alien genes for resistance from wild species provide the way for sustaining crop improvement through pre-emptive breeding. Available resistance in wild *Cicer* species and their utilization to overcome various biotic and abiotic stresses and other useful traits are described below.

4.6.1 Ascochyta Blight

Among the foliar fungal diseases, Ascochyta blight, caused by *A. rabiei*, is the most devastating disease of chickpea. It is reported to occur in as many as 30 countries (Singh and Reddy 1991). It causes serious yield losses in India, Pakistan, and the countries around the Mediterranean region, where cool and humid climates prevail. Crop loss due to the disease can be 100% (Nene 1982). Under favorable weather conditions, the disease takes epidemic proportions. Such epidemics

have occurred in India, Pakistan, the US, Pacific northwest, Australia, and Syria (Malhotra et al. 2003). More than 60 years of efforts in managing the disease through cultural practices, chemical control, and exploitation of host plant resistance available in the cultivar have not resulted in satisfactory control of the disease. The reason for the failure of the elite resistant genotypes developed from intraspecific crosses within chickpea is attributed to the low levels of resistance caused by the development of more virulent pathotypes in different chickpea-growing regions, for example, Italy (Stamigna et al. 2000), Syria, Lebanon (Reddy and Kabbabeh 1985), and Pakistan (Jamil et al. 2000).

Several wild species are identified to be resistance sources for Ascochyta blight in chickpea, such as *C. reticulatum* and *C. echinospermum* (Collard et al. 2001). A linkage map was constructed based on an interspecific F_2 population derived from the cross between cultivated chickpea and *C. echinopsermum* (PI 527930; Collard et al. 2003). Santra et al. (2000) reported two quantitative trait loci (QTLs), QTL1 and QTL2, that confer resistance to Ascochyta blight in US. These QTLs accounted for an estimated 34.4% and 14.6% of the total phenotypic variance (Santra et al. 2000; Tekeoglu et al. 2000, 2002). Two QTLs were identified for seedling resistance and five markers were associated with stem resistance, four of which were also associated with seedling resistance in Australia (Collard et al. 2003). Udupa and Baum (2003) identified a single major gene conferring resistance to the pathotype 1 and two QTLs for pathotype II. Rakshit et al. (2003) mapped OPS06, a DNA amplification fingerprinting (DAF) marker, between the flanking markers at QTL1 of Santra et al. (2000) along with several other DAF markers.

The environmental effect of Ascochyta blight QTL1 and QTL2 was analyzed at Eskisehir, Turkey (Tekeoglu et al. 2004), using CRIL-7 (Santra et al. 2000) developed in the USA from the interspecific cross FLIP84-92C × PI 599072. The same two QTLs were identified at both the locations indicating their robust nature, with some differences. The effect of QTL1 was greater than QTL2 at Pullman, Washington, USA, whereas the effect of QTL2 was greater than QTL1 at Eskisehir, Turkey, indicating possible differences in pathogen population and environmental interactions (Tekeoglu et al. 2004). Irula et al. (2006) used an intraspecific population developed from the cross ILC3279 × WR315 and identified two QTLs that were the same as that of Santra et al. (2000). More recently, Pande et al. (2006) identified moderate levels of resistance in accessions belonging to *C. cuneatum*, *C. pinnatifidum*, *C. judaicum*, and *C. bijugum,* and accessions of *C. judaicum* and *C. bijugum* showed higher levels of resistance. Some accessions of *C. echinospermum* have also shown resistance to Ascochyta blight (Pande et al. 2006).

4.6.2 Botrytis Gray Mold

Botrytis gray mold (BGM) is prevalent in 14 countries, including the three main chickpea-growing countries, India, Pakistan, and Turkey. The disease occurs regularly but increased damage occurs when there are rains and high humidity during flower/pod formation. Screening of chickpea germplasm and breeding lines in India and Nepal has failed to identify high levels of resistance (Singh and Reddy 1991). Among the wild species, resistance has been identified in *C. bijugum* (Haware et al. 1992), *C. pinnatifidum*, and *C. judaicum* (van der Maesen and Pundir 1984). More recently, Pande et al. (2006) identified BGM resistance in *C. echinospermum* accessions, apart from *C. bijugum*, *C. pinnatifidum*, and *C. judaicum* accessions. It was possible to transfer BGM resistance from *C. echinospermum* to the cultigens, and the nature of resistance was found to be monogenic and recessive (Mallikarjuna unpublished results). Stevenson and Haware (1999) attributed resistance in *C. bijugum* to BGM with high concentrations of chemical maackiain (200–300 $\mu g/g^{-1}$) compared to low concentrations of maackiain (70 $\mu g/g^{-1}$) in susceptible wild and cultivated species.

4.6.3 Fusarium Wilt

Fusarium wilt (*F. oxysporum f.* sp. *Ciceri*) is a major constraint to chickpea production and yield losses due to wilt have been estimated at 10–90% (Jimenez-Diaz et al. 1989; Singh and Reddy 1991). The pathogen persists in soil year after year even in the absence of the host, which renders its control difficult (Haware et al. 1996). Vertical resistance to wilt is available in

cultivated chickpea (Sharma et al. 2005). There are eight distinct physiological races of wilt namely 0, 1A, 1B/C, 2, 3, 4, 5, and 6 (Haware and Nene 1982; Jimenez-Diaz et al. 1993; Kelly et al. 1994). The pathogen exhibits variation with respect to occurrence, regional specificity, and disease symptoms (Sharma et al. 2009). Races 0 and 1B/C cause yellowing syndrome, whereas 1A, 2, 3, 4, 5, and 6 lead to wilting syndrome. Genetics of resistance to two races (1B/C and 6) is yet to be determined; however, for other races, resistance is governed either by monogenes or by oligogenes. The individual genes of the oligogenic resistance mechanism delay the onset of disease symptoms, a phenomenon called as late wilting. Slow wilting, i.e., slow development of disease after onset of disease symptoms also occurs in reaction to pathogen; however, its genetics is not known.

STMS markers have revealed significant interspecific and intraspecific polymorphism in chickpea. Markers linked to six genes that govern resistance to six races (0, 1A, 2, 3, 4, and 5) of the pathogen have been identified and their position on chickpea linkage maps elucidated. These genes lie in two separate clusters on two different chickpea linkage groups. While the gene for resistance to race 0 is situated on linkage group (LG 5) of Winter et al. (2000), those governing resistance to races 1A, 2, 3, 4, and 5 spanned a region of 8.2 cm on LG 2. The cluster of five resistance genes was further subdivided into two subclusters of 2.8 cm and 2.0 cm, respectively.

Wild relative *C. judaicum* roots have three isoflav-3-enes, together with two pterocarpan glycosides. Initial experiments have shown that these chemical compounds may confer resistance to Fusarium wilt fungi (Stevenson and Veitch 1996). Later, these compounds were isolated in many annual and perennial *Cicer* species (Stevenson and Veitch 1998).

4.6.4 H. armigera *(Pod Borer)*

The legume pod borer [*H. armigera* (Hubner)] is an important pest of chickpea globally and brings down the yield of the crop. Losses due to *H. armigera* are estimated at US$927 million on chickpea and pigeonpea worldwide (Gowda 2005). It is polyphagous insect and attacks more than 182 plant species. The levels of resistance to pod borer in cultivated chickpea is not up to the desired level and other management options such as biological and chemical control have their own limitations. Therefore, development of host plant resistance is the best option as this can be coupled with management options.

Resistance to pod borers has been reported in wild *Cicer* species, namely, in accessions of *C. bijugum*, *C. pinnatifidum*, *C. judaicum*, *C. reticulatum*, and *C. echinospermum* (Sharma et al. 2005a). Mallikarjuna et al. (2007a) utilized *C. reticulatum* and *C. echinospermum* and obtained progeny that consistently showed low field damage (10% or less) due to pod borers. Laboratory bioassay using third instar larvae fed on the pods of resistant plants showed reduced larval weight, delayed pupation, failure to pupate or death before pupation, and in some cases, abnormal adults. This shows that antibiosis mechanism of resistance exists in these wild *Cicer* species, which can be transferred and exploited in a breeding program to develop cultivars with resistance against the insect.

Simmonds and Stevenson (2001) identified for the first time four isoflavonoids, namely, judaicin 7-0-gluoside, 2-methoxy judaicin, maackiain, and judaicin, which deterred larval feeding by *H. armigera* at 100 ppm concentration. Flavonoids judaicin and maakiain retained their antifeedant activity at 50 and 10 ppm, respectively. Additionally, chlorogenic acid increased their antifeedant potency. These flavonoids may be the substances responsible for antibiosis mechanism of resistance observed by Mallikarjuna et al. (2007a). Antibiosis mechanism of resistance to *Spodoptera litura* was observed in interspecific progeny derived from wild species *Arachis kempff-mercadoi* with the presence of chlorogenic acid, quercetin, and rutin (Mallikarjuna et al. 2004). Flavonoids chlorogenic acid, quercetin, and rutin were present in larger quantities in *A. kempff-mercadoi* (Stevenson et al. 1993) than in the susceptible cultivated groundnut, and these substances were responsible for conferring resistance to *S. litura*.

4.6.5 Bruchids (Callosobruchus chinensis)

Many storage insects, specifically bruchids, are a serious pest of stored chickpea (Southgate 1978). Chickpeas stored as “dhal” harbor fewer bruchids than when

stored as whole grains. Bruchids lower seed viability. For control of bruchids, dusting with BHC, DDT, derris, lindane, or pyrethrum or fumigation with methyl bromide has been recommended (Duke 1981). Resistance to seed beetles is not available in chickpea genotypes (Di Vito et al. 1988). At ICARDA, 127 accessions of eight wild *Cicer* species were screened for resistance to seed beetles (Singh et al. 1994, 1998). Three accessions of *C. echinospermum*, six accessions of *C. judaicum*, and nine accessions of *C. bijugum* were free from pest damage. It is possible that some of the flavonoids present in wild *Cicer* species (Simmonds and Stevenson 2001) may be responsible for bruchid resistance. There is no report of successfully transferring bruchid resistance from wild *Cicer* species to cultivated forms.

4.6.6 Cyst Nematode (H. ciceri *Vovlas, Greco, and Divito)*

Cyst nematode is an important pest in West Asia and North Africa and causes heavy losses to chickpea production (Greco et al. 1988). Resistance to cyst nematode has been identified in accessions of *C. bijugum*, *C. pinnatifidum* and in one accession of *C. reticulatum* ILWC 119. Chickpea germplasm lines, ILC 10765 and ILC 10766, resistant to cyst nematode were derived from crosses utilizing *C. reticulatum* ILWC 119 (Malhotra et al. 2002).

4.6.7 High Protein Content and High Yield

Significant variation in seed protein content has been observed in wild *Cicer* species, with some of the species showing higher content compared with cultivated chickpea (Singh and Pundir 1991). Ocampo et al. (1998) are of the opinion that this may be due to the methodology used in the estimation. Utilization of wild relatives in sorghum (Cox et al. 1984), soybean (Li et al. 2008), and rice (McCouch et al. 2007) has shown that wide crosses could produce positive transgressive segregants with high yield.

Although the present-day chickpea cultivars have been developed to produce more than the traditional varieties, there is ample scope to increase the yield, as there is a gap between potential yield and the actual yield obtained. In order to introduce yield genes into chickpea cultivars, chickpea cultivars were crossed with *C. reticulatum* and *C. echinospermum* on the premise that recombination could result in progenies with high yield. Heterosis was visually recorded in F_1 plants and promising and uniform progenies were bulked in F_5. Lines with higher yield (39%) than the controls were observed in F_7 (Singh and Ocampo 1997).

4.6.8 Cold Tolerance

Cold conditions result in flower drop in chickpea, culminating in significant yield loss in the semi-arid tropics (Malhotra et al. 1997). In the Mediterranean region, winter sowing is more productive compared to traditional sowing in spring, and cold tolerance is an important prerequisite for winter sowing (Singh and Hawtin 1979). Many accessions of C. *bijugum*, one accession of *C. echinospermum*, 13 accessions of *C. reticulatum*, and one accession of *C. pinnatifidum* showed higher levels of cold tolerance than the cultivated species (Singh et al. 1990). There are no reports on transfer of cold tolerance from wild *Cicer* species to the cultigen.

4.6.9 Drought Tolerance

Chickpea is sensitive to water stress during the early pod development (Khanna-Chopra and Sinha 1987). As the crop is mostly grown under residual moisture situation, scanty and early cessation of rains can cause significant yield losses in chickpea. Recently, seven annual wild *Cicer* species were investigated for their root traits along with chickpea genotypes (Krishnamurthy et al. 2003). The root and shoot growth of annual wild *Cicer* species was relatively poor compared to cultivated chickpea genotypes. Among the annual wild *Cicer* species, *C. reticulatum* showed growth rates closer to cultivated genotypes. In a study, Canci and Toker (2009) reported a few accessions of *C. reticulatum* and *C. pinnatifidum* to perform better under drought conditions and those lines could be considered as the best available drought-resistant

sources for breeding purposes. Utilizing *C. reticulatum* in place of cultivated chickpea would bring in much desired genetic variation in the resultant population. Toker et al. (2007) also found drought-resistant accessions of *C. pinnatifidum* and *C. reticulatum* on par with currently available resistant sources. Some of the perennial *Cicer* species not only recovered after wilting and drying out above the ground level but also tolerated temperatures above 41.8°C. A dehydrin gene (*cpdhn1*) related to drought resistance was isolated from a cDNA bank prepared from ripening seeds of *C. pinnatifidum* (Bhattarai and Fettig 2005). Two perennial species, *C. microphyllum* and *C. montbretii*, have been reported to be drought tolerant, inferred from their distribution in alpine regions (Chandel 1984). Additionally, *C. stapfianum*, *C. subaphyllum*, and *C. pungens* have been found to be drought resistant based on their growing region (van der Maesen, personal communication). Most perennial *Cicer* species are known to have a long woody tap root, which can often penetrate to 2 m depth. Long root system is marked as an important trait for drought tolerance. This trait may be useful in chickpea, which is often grown under receding moisture conditions. Although systematic screening of perennial wild *Cicer* species to quantify the variation in root growth is required for their exploitation in crop improvement programs, it can be concluded on the basis of the available information that perennial *Cicer* species are good sources of drought tolerance/resistance.

4.7 Genomics Resources

The chickpea genome is considered homogeneous, based on the minimal polymorphism detected by molecular markers. Limited polymorphism may be due to the self-pollinating nature. Although RAPDs were used initially, the development of highly variable polymorphic SSR markers has replaced them. Linkage maps were developed using amplified fragment length polymorphism (AFLPs), RAPDs, ISSRs, RGAs (resistance gene analogs), and STMS markers (Santra et al. 2000; Winter et al. 2000; Cho et al. 2002, 2004; Collard et al. 2003; Flandez-Galvez et al. 2003). Development of numerous SSR markers has accelerated chickpea genomics and the study of important traits. In chickpea, several hundred SSR markers have been developed and mapped on both intra- and interspecific mapping populations (Huttel et al. 1999; Winter et al. 1999; Lichtenzveig et al. 2005; Sethy et al. 2006). Functional markers generated from genic sequence, expressed tag sequences (ESTs), are advantageous as they are linked to traits of interest. Buhariwalla et al. (2005) developed 106 EST markers, of which 14 contained SSR motifs, and these were the first chickpea EST-SSR markers. More recently, Choudhary et al. (2009) generated 822 ESTs from immature seeds as well as 1,309 ESTs from chickpea database. From these, 246 SSR motifs were identified and 60 were validated as functional markers. These markers showed low levels of intraspecies polymorphism and high level of interspecies polymorphism.

The complete plastid genome of chickpea was sequenced by Jansen et al. (2008), and it was found to be 125,319 bp in size. The genome encodes 108 genes, including four rRNAs, 29 tRNAs, and 75 proteins. The sequence provides valuable information on the intergeneric spacer regions among legumes and endogenous regulatory sequences for plastid genetic engineering.

Jayashree et al. (2005) reported the development of a chickpea root-specific EST database comprising of over 2,800 EST sequences. This was constructed from using substractive suppressive hybridization (SSH) of root tissues from two closely related chickpea genotypes possessing different sources of drought avoidance and tolerance. The database provides researchers in chickpea genomics with a major resource for data mining association with root traits and drought tolerance.

Development of diversity array technology (DArT) markers should overcome the constraint associated with the development of high-density linkage maps. The discovery of single nucleotide polymorphism (SNP), which is relatively new in plant systems, has great potential for marker development. Rajesh and Muehlbauer (2008) estimated single nucleotide polymorphism (SNP) frequency at 1 in 94 bp in coding sequences and 1 in 74 bp in genomic regions in chickpea line FLIP 84-92C and wild relative *C. reticulatum* (PI 599072), two parental chickpea lines previously used to develop an interspecific linkage map.

A strategy for reverse genetics that is based on ethylmethyl sulfonate (EMS) mutagenesis was first described by McCallum et al. (2000) using the acronym targeted induced local lesions in genomes (TILLING). A specific advantage of EMS mutagenesis

is that the series of allelic mutations can serve as the basis of detailed structure-function studies. In addition, this has the potential to recover weak alleles with subtle changes in functionality of genes that would be lethal when more strongly affected. TILLING identifies individuals carrying point mutations in any gene of interest within a large population of EMS-mutagenized plants. In chickpea, M_2 seeds from approximately 9,000 individual M_1 plants of chickpea germplasm accession ICC12004 that had been treated with 0.2% EMS were obtained in the initial phases for development of a TILLING platform for chickpea. The estimated mutation frequency was determined through an analysis of 768 M_2 progenies using 20 targets comprising genomic DNA and cDNA sequences. There was a 100% success rate in primer design and screening of the mutants when using genomic sequence, and only a 7% success rate using cDNA sequence (Muehlbauer and Rajesh 2008).

4.7.1 Molecular Maps

Progress has been made in the development of genetic maps and the placement of genes for resistance to Ascochyta blight and Fusarium wilt as well as genes controlling agronomically important traits such as time to flowering, time to maturity, and podding habits. Two types of mapping populations have been used in chickpea to generate genetic linkage maps: the F_2 population and recombinant inbred lines (RILs) derived from interspecific as well as intraspecific crosses. RILs are preferred for genome mapping because of the distinct advantages it offers. The first integrated molecular map with 354 markers, including 118 STMSs, 96 DAFs, 70 AFLPs, 37 ISSRs, 17 RAPDs, eight isozymes, three cDNAs, and two sequence-characterized amplified regions (SCARs),which covered a distance of 2,077.9 cm was the result of an international collaborative effort (Winter et al. 2000).

Santra et al. (2000) used an RIL population from an interspecific cross of *C. areitinum* × *C. reticulatum* to generate a map of nine linkage groups with 116 markers (isozymes, RAPDs, and ISSRs) covering a map distance of 981.6 cm with an average distance of 8.4 cm between markers. In order to identify blight resistance in chickpea as well as genomic regions associated with blight resistance on intraspecific genetic linkage maps, F_7-derived RILs from the intraspecific cross of PI 359075 (blight susceptible) × FLIP84-92C(2) (blight resistant) were used by Cho et al. (2004). An intraspecific genetic linkage map comprising 53 STMS markers was constructed to identify genomic associations with blight resistance on the RIL population from a cross between PI359075(1) and FLIP84-92C(2). A major QTL for resistance to pathotype II of *A. rabiei* and two QTLs for resistance to pathotype I were identified.

Flandez-Galvez et al. (2003) established an intraspecific linkage map of chickpea genome using STMS markers on a F_2 population of chickpea cultivars with contrasting reaction to Ascochyta pathogen. Fifty-one out of 54 STMS markers (94.4%), three ISSR markers (100%), and 12 resistance gene analog (RGA) markers (57.1%) mapped on eight linkage groups. Chickpea-derived STMS markers were distributed throughout the genome, while RGA markers clustered with ISSR markers on the linkage groups LG 1, II, and III. Intraspecific linkage map spanned 534.5 cm, with an average interval of 8.1 cm between markers.

Madrid et al. (2008) identified a gene that controls resistance to chickpea rust in an RIL population derived from an interspecific cross between chickpea and *C. reticulatum*. A QTL for 31% of the phenotypic variance was located on the LG 7 of the chickpea genetic map. Two STMS markers were detected flanking the resistance gene.

A composite linkage map was constructed using RILs from a cross between *C. arietinum* and *C. reticulatum*. The mapping population segregated for resistance to Ascochyta blight, Fusarium wilt, and rust diseases. RGA markers have mapped loci that confer resistance to Ascochyta blight and Fusarium wilt. Association was detected between RGAs and genes that controlled resistance to Fusarium wilt caused by races 0 and 5 (Palomino et al. 2009).

The genetic map published by Taran et al. (2007) was generated from 135 primer pairs including 134 SSRs and was based on a population of 186 F_2 plants from an intraspecific cross of "desi" cultivar ICCV 96029 and "kabuli" cultivar CDC Frontier. Markers reported in this map were assigned to eight linkage groups with a combined linkage distance of 1,285 cm. The average linkage distance between markers in all linkage groups was 8.9 cm. Common markers in these maps with SSR primer pairs could lead to the development of a high-density genetic map of chickpea to

identify tightly linked flanking markers for genes of interest, which will ultimately be helpful in marker-assisted selection (MAS) and positional cloning of agronomically important genes.

4.8 Directions for Future Research

Taking examples from rice and wheat, it is clear that a particular wild *Cicer* species may not possess many of the desirable traits needed for chickpea improvement. One of the major advantages of utilizing wild relatives is the resultant reshuffling/recombination in the genome, leading to the appearance of novel characters not found in parental species. With a large collection of molecular markers available for chickpea, it may not be too difficult to select against unwanted characters from the wild species brought along because of linkage drag.

Several wild species are poorly represented in gene banks either because of developmental activities in the areas of their origin or because of the inaccessibility of the region due to geographical or political reasons. Collection of additional accessions of each annual and perennial wild species is needed to widen the genetic base in the *Cicer* gene pool. Specifically, major emphasis should be placed on collection of *C. reticulatum* in the center of origin of chickpea, most importantly in Turkey, where the species is found in abundance. Collection could be followed by transfer of the agronomically important genes such as Ascochyta blight, botrytis gray mold, *H. armigera* to the cultigen by classical breeding methods. It will be useful if focused attempts in wide crosses are initially de-linked from the active breeding programs (often termed as pre-breeding) to utilize the available variation in the wild species. This will remove the pressure of agronomic performance for the segregants from the wide crosses. Although variation in resistance to some of the stresses is available in chickpea, it is not up to the desired level, and the use of genes from the wild species will broaden their genetic base and likely improve stability of resistance. Increased emphasis also needs to be placed on the collection of *C. echinospermum* as some of the accessions have shown variation for economically important traits. It is important to evaluate the available wild gene pool for various biotic and abiotic constraints to tap the potential of the available germplasm.

Low biomass production is often cited as one reason for low seed yields of chickpea. Increasing biomass of cultivated chickpea varieties appears to be possible with the use of *C. reticulatum* and *C. echinospermum* as progenies of both species in crosses with *C. arietinum* have had an apparent increase in biomass and overall plant vigor. Perennial *Cicer* also has potential for higher biomass but currently is unavailable for breeding purposes due to cross-incompatibility with the cultigen.

Advanced molecular marker technology should be exploited to estimate genetic diversity and phylogenetic relationships among annual and perennial wild species. Molecular markers may also improve our genetic understanding of the traits, and genetic mapping and QTL analysis will provide useful information on the locations of important genes and markers that can be used for gene introgression. The desired genes can eventually be transferred from wild species to the cultigen using marker-assisted breeding, which will ultimately facilitate chickpea crop improvement.

The presence of biochemical compounds with antifungal activity toward botrytis gray mold and antifeedant activity toward *H. armigera* opens up avenues for molecular breeding for fungal and insect resistance in chickpea coupled with biochemical markers.

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Chapter 5
Glycine

M.B. Ratnaparkhe, R.J. Singh, and J.J. Doyle

5.1 Introduction

Legumes (Fabaceae or Leguminosae) are the third largest family of angiosperms with 730 genera and more than 19,000 species distributed throughout the world (Lewis et al. 2005). Traditionally, the legume family has been divided into three subfamilies: Caesalpinieae, Mimosoideae, and Papilionoideae, of which only the latter two are natural, with "caesalpinioid" legumes comprising several unrelated groups (Lewis et al. 2005; Bruneau et al. 2008). The grain legumes are included in the Papilionoideae. Within that subfamily, there are four large clades that include most of the economically important food and feed legumes (Doyle and Luckow 2003; Wojciechowski et al. 2004; Lewis et al. 2005) including *Glycine*. The genus *Glycine* is formally classified in the large tribe Phaseoleae, which also includes several other economically important genera such as *Phaseolus* (e.g., common bean and scarlet runner bean), *Vigna* (cowpea, mungbean), *Cajanus* (pigeon pea), *Psophocarpus* (winged bean), and *Pachyrhizus* (jicama) (Lackey 1977a). Most Phaseoleae are part of a clade of phaseoloid legumes that also includes the tribes Psoraleeae and Desmodieae (e.g., the forage crop, *Lespedeza*); this clade is part of a larger "millettioid" group of genera that includes such genera as *Lonchocarpus* and *Derris*, used by indigenous people in both the eastern and western Hemispheres as fish poisons (Kajita et al. 2001; Wojciechowski et al. 2004). Within the phaseoloid clade, *Glycine* is part of a clade that includes most of the genera formally classified as subtribe Glycininae (Lackey 1977a, c; Polhill 1994). The closest relatives of *Glycine*, based on chloroplast DNA phylogenies, are *Teramnus*, *Amphicarpaea* (hog-peanut, whose tubers were eaten by indigenous peoples of North America), and the tribe Psoraleeae (Stefanovic et al. 2009). These genera diverged from one another around 10 million years ago (Stefanovic et al. 2009).

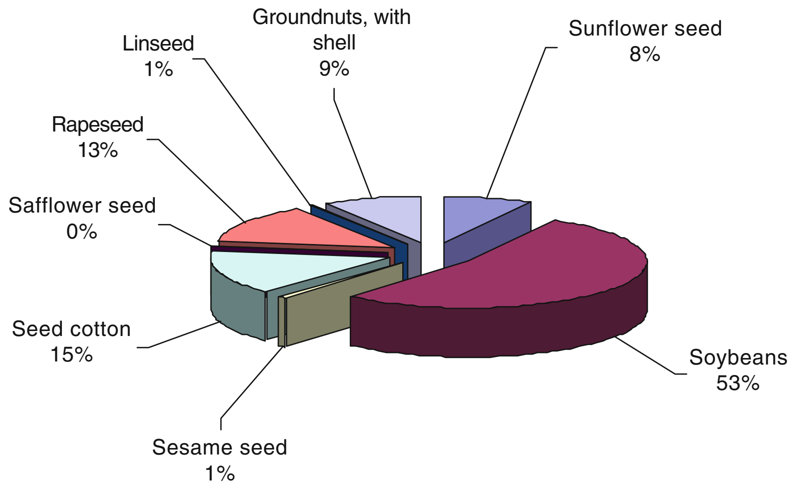
The genus *Glycine* is divided into two subgenera, *Glycine* Willd. (perennial) and *Soja* (Moench) F.J. Herm (annual). The subgenus *Soja* includes two species: an economically very important crop, the soybean [(*G. max* (L.) Merr.)], and its wild annual progenitor, *G. soja* Sieb. and Zucc. The two species are cross-compatible and producing vigorous and fertile F_1 hybrids, suggesting that they have similar genomes (Singh and Hymowitz 1988). The subgenus *Glycine* contains 26 wild perennial species, which are indigenous to Australia, diverse morphologically, cytologically, and genomically, and grow in a wide range of agroclimatic regions of Australia (Chung and Singh 2008).

Soybean is rich in seed protein (about 40%) and oil (about 20%) and enriches the soil by fixing nitrogen through symbiosis with bacteria. In the international world trade markets, soybean is ranked number one (53%) among the major oil crops such as rapeseed, groundnut (peanut), cottonseed, sunflower seed, linseed, sesame seeds, and safflower (Fig. 5.1; FAOSTAT 2008; *http://www.fao.org*). Even though soybean is a very valuable legume crop, it is not considered a model plant for cytogenetic studies because of the large number of chromosomes ($2n = 40$) (Karpechenko 1925; *Soja hispida*, syn. *G. max*), their small and similar chromosome size (1.42–2.84 μm; Sen and Vidyabhusan 1960), and

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Fig. 5.1 The world production of eight major oilseed crops (2008) (*http://faostat.fao.org*)



lack of morphological distinguishing landmarks (Ladizinsky et al. 1979; Singh 2003). Using primarily restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) marker loci, 20 molecular linkage groups (MLGs) have been developed (Song et al. 2004; Xia et al. 2007), however the cytogenetic knowledge of the soybean lags far behind many crops such as maize, barley, rice, wheat, tomato, Brassicas, pea, and faba bean (Singh 2003; Zou et al. 2003; Singh et al. 2007a, b).

5.2 Basic Botany of the Species

5.2.1 Taxonomy

The taxonomy of cultivated soybean is as follows:

Order	Fabales
Family	Fabaceae (Leguminosae)
Subfamily	Papilionoideae
Tribe	Phaseoleae
Subtribe	Glycininae
Genus	*Glycine* Willd.
Subgenus	*Soja* (Moench) F. J. Herm.
Botanical name	*Glycine max* (L.) Merr.

The taxonomy of the genus *Glycine* to which soybean belongs has been revised many times. Hermann (1962) divided the genus *Glycine* into three subgenera (Table 5.1): *Leptocyamus* (Benth.) F. J. Herm. with six wild perennial species indigenous to Australia and the Pacific Islands, *Glycine* L. with two species of African origin, and *Soja* (Moench) F. J. Herm. containing soybean [*G. max* (L.) Merr.] and *G. ussuriensis* Regel and Maack. Table 5.2 shows the revision of the genus *Glycine* by Verdcourt (1966). Verdcourt (1970) questioned the validity of *G. javanica* L. as it has $2n = 22$ or 44 chromosomes, and the chromosomes (morphology) are larger than those of other species of the genus *Glycine*. He kept the generic name and proposed *G. wightii* (R. Grah. Ex Wight and Arn.) Verdcourt as the species name. He changed the names of the genus *Glycine* L. to *Glycine* Willd. and the names of two of the subgenera of *Glycine*: subgenus *Leptocyamus* (Benth.) Hermann became a synonym of *Glycine* subgenus *Glycine*; subgenus *Soja* (Moench) Hermann was unchanged. Lackey (1977b) later removed *G. wightii* (Arnott) Verdcourt from the genus *Glycine* and designated it *Neonotonia wightii* (Wight and Arn.) J.A. Lackey, because it has $2n = 22$ or 44 large mitotic metaphase chromosomes and canavanine (non-proteinogenic amino acid) in seeds.

Table 5.3 shows the current information on the taxonomy of genus *Glycine*. The species in the subgenus *Glycine* are distributed in Australia and various South and West Pacific Islands (Hymowitz and Singh 1987; Doyle et al. 2003; Chung and Singh 2008). Of the 26 perennial species, *Glycine tomentella* Hayata constitutes four cytotypes ($2n = 38, 40, 78, 80$), and *G. hirticaulis* Tindale and Craven,and *G. tabacina* (Labill.) Benth. have accessions with $2n = 40$ and 80 chromosomes, respectively (Table 5.3). *Glycine tomentella* accessions with $2n = 80$ chromosomes with A-, D-, and E-genomes were distributed in

Table 5.1 Systematic classification of the *Glycine* species L. (Hermann 1962)

Species	Distribution
Subgenus *Leptocyamus*	
Glycine clandestina Wendl.	Australia; Formosa (Taiwan), Micronesia
var. *sericea* Benth.	Australia
G. falcata Benth.	Australia
G. latrobeana (Meissn.) Benth.	Australia
G. canescens F. J. Herm.	Australia
G. tabacina (Labill.) Benth.	Australia; S. China; S. Pacific Islands
G. tomentella Hayata	Australia; S. China; Philippines; Formosa (Taiwan)
Subgenus *Glycine*	
G. petitiana (A. Rich.) Schweinf.	Ethiopia
G. javanica L.	India; Malaya (Malaysia)
ssp. micrantha (Hochst.) F.J.Herm.	Tropical Africa
var. *claessensii* (De Wild.) Hauman	Uganda to Nyasaland (Republic of Malawi)
var. *paniculata* Hauman	Belgian Congo (Democratic Republic of the Congo)
var. *longicauda* (Schweinf.) Bak.	Ethiopia to Angola
var. *moniliformis* (Hochst.) F.J.Herm.	Ethiopia to Eritrea
subsp. *pseudojavanica* (Taub.) Hauman	Belgian Congo (Democratic Republic of the Congo) to Angola
var. *laurentii* (De Wild.) Hauman	Belgian Congo (Democratic Republic of the Congo)
Subgenus *Soja*	
G. ussuriensis Regal&Maack.	Asia
G. max (L.) Merr.	Cultigen

Table 5.2 Revision of the genus *Glycine* by Verdcourt (1966)

Genus *Glycine* Willd.
Subgenus *Glycine*
Glycine clandestina Wendl.
var. *sericea* Benth.
G. falcata Benth.
G. latrobeana (Meissn.) Benth.
G. canescens F. J. Herm.
G. tabacina (Labill.) Benth.
G. tomentella Hayata
Subgenus *Bracteata* Verdcourt
G. wightii (R.Grah. ex Wight and Arn.) Verdcourt
Subgenus *Soja*
G. soja Sieb. and Zucc.
G. max (L.) Merr.

Australia, Papua New Guinea, the Philippines, Indonesia, Miyako Island of Japan, and southern China, including Fujian and Taiwan, while 80-chromosome *G. tabacina* has been collected from Australia, Tonga, Vanuatu, Ryukyu Islands, and Taiwan. Tateishi and Ohashi (1992) examined *Glycine* of Taiwan and recognized four species: *G. dolichocarpa* Tateishi and Ohashi, *G. tomentella*, *G. tabacina*, and *G. max* subsp. *formosana* (Hosokawa, Tateishi and Ohashi). *Glycine dolichocarpa* contains $2n = 80$ chromosomes, and *G. tomentella* and *G. tabacina* of Taiwan contain $2n = 80$ chromosomes (Singh unpublished results). Subsequent study has shown that the tabacina complex consists of two allopolyploid species (*G. tabacina* and *G. pescadrensis*) that share one of their two genomes; both species are found in Taiwan (Doyle et al. 1990c, 1999a, b, 2000). *G. dolichocarpa* is "*G. tomentella* T2" of Doyle et al. (2002). All of these polyploids are thought to have arisen in Australia, and migratory birds are thought to have been responsible for their dispersal throughout the Pacific (Hymowitz et al. 1990).

The subgenus *Soja* includes soybean, cultigen (*G. max*), and its wild progenitor *G. soja* Sieb. and Zucc. Both species are annual, contain $2n = 40$ chromosomes, are cross-compatible, and produce fertile F_1 plants, and gene exchange between them is possible. Broich and Palmer (1980) used cluster analysis techniques to examine phenotypic variation among *G. max*, *G. soja*, and *G. gracilis*. *Glycine max* and *G. soja* were found to be morphologically distinct, and *G. gracilis* was found to be conspecific with *G. max*. Thseng et al. (1999) identified a new species *G. formosana* Hosokawa from Taiwan based on pod morphology, allozyme, and DNA polymorphisms, and concluded that the newly defined species is different from *G. soja*, though they did not hybridize both species. It is likely that *G. formosana* is a variant of *G. soja*. Thus, we have not included *G. formosana* in Table 5.3.

5.2.2 Morphology

Cultivated and wild soybeans differ in a set of various morphological and physiological characteristics collectively designated as the domestication syndrome (Broich and Palmer 1980, 1981). The typical

Table 5.3 Taxonomy of the Genus *Glycine* Willd

Species	Mol. group	2*n*	Genome Symbol N	C	PI-number	G number	Distribution	Species described since Tables 5.1 and 5.2
Subgenus *Glycine*								
G. albicans Tindale and Craven		40	I	A	–	2049	Aust. : WA	Tindale and Craven (1988)
G. aphyonota B. Pfeil		40	I_3	A		2589	Aust. : WA	Pfeil and Craven (2002)
G. arenaria Tindale		40	H	A	505204	1305	Aust. : WA	Tindale (1986b)
G. argyrea Tindale		40	A_2	A	505151	1420	Aust. : Q	Tindale (1984)
G. canescens F.J. Hermann		40	A	A	440932	1853	Aust. : Q, NSW, V, SA, NT, WA	
G. clandestina Wendl.		40	A_1	A	440958	1126	Aust. : Q, NSW, V, SA, T	
G. curvata Tindale		40	C_1	C	505166	1849	Aust. : Q	Tindale (1986a)
G. cyrotoloba Tindale		40	C	C	440962	1184	Aust. : Q, NSW	Tindale (1984)
G. falcata Benth.		40	F	A	505179	1155	Aust. : Q, NT, WA	
G. gracei B.E. Pfeil and Craven		40	?	?		3124	Aust. : NT	Pfeil et al. (2006)
G. hirticaulis Tindale and Craven		40	H_1, (??)	A, (A)	IL1246	2876	Aust. : NT	Tindale and Craven (1988)
		80			IL943	1956	Aust. : NT	
G. lactovirens Tindale and Craven		40	I_1	A	IL1247	2720	Aust. : WA	Tindale and Craven (1988)
G. latifolia (Benth.) Newell and Hymowitz		40	B_1	B	378709	1697	Aust. : Q, NSW	Newell and Hymowitz (1980)
G. latrobeana (Meissn.) Benth.		40	A_3	A	483196	1385	Aust. : V, SA, T	
G. microphylla (Benth.) Tindale		40	B	B	440956	1867	Aust. : Q, NSW, V, SA, T	Tindale (1986b)
G. montis-douglas B.E.Pfeil and Craven		40	?	?			Aust. : NT	Pfeil et al. (2006)
G. peratosa B.E. Pfeil and Tindale		40	A_5	A		2916	Aust. : WA	Pfeil et al. (2001)
G. pescadrensis Hayata		80	AB_1	A	440996	1433	Aust. : Q, NSW; Taiwan, Japan	Pfeil et al. (2006)
G. pindanica Tindale and Craven		40	H_2	A	595818	2951	Aust. : WA	Tindale and Craven (1993)
G. pullenii B. Pfeil, Tindale and Craven		40	H_3	A		2599	Aust. : WA	Pfeil and Craven (2002)
G. rubiginosa Tindale and B. E. Pfeil		40	A_4	A	440954	1874	Aust. : NSW, SA, WA	Pfeil et al. (2001)
G. stenophita B. Pfeil and Tindale		40	B_3	B	378705	2600	Aust. : Q, NSW; (Japan ??)	Doyle et al. (2000)
G. syndetika B.E. Pfeil and Craven	D4	40	A6		441000	1300	Aust. : Q	Pfeil et al. (2006)
G. dolichocarpa Tateishi and Ohashi		80	D_1A	?			Taiwan	Tateishi and Ohashi (1992)
G. tabacina (Labill.) Benth.		40	B_2	B	373990	1317	Aust. :Q, NSW	
		80	BB_1;BB_2;B_1B_2	B	373992	1314	Aust. :Q, NSW, V, SA; West Central and South Pacific Islands[a]	

(*continued*)

Table 5.3 (continued)

Species	Mol. group	2*n*	Genome Symbol N	C	PI-number	G number	Distribution	Species described since Tables 5.1 and 5.2
G. tomentella Hayata	D1, D2	38	E	A	440998	1858	Aust. : Q	
	D3	40	D	A	505222	1749	Aust. : Q, WA, PNG	
	D5B	40	H_2	A	505294	1943	Aust. : WA	
	D5A	40	D_2	A	505203	1303	Aust. : WA, NT	
	T1	78	D_3E	A	441001	1133	Aust. : Q, NSW; PNG	
	T5	78	AE	A	509501	1487	Aust. : NSW	
	T6	78	E H_2	A	505286	1945	Aust. : WA	
	T2	80	D A_6	A	441005	1188	Aust. : Q; Taiwan	
	T3	80	D D_2	A	483219	1927	Aust. : Q, NT, WA; PNG; Timor	
	T4	80	D H_2	A	330961	1348	Aust. : Q, NT, WA; Philippines, Taiwan	
Subgenus Soja (Moench) F. J. Hermann								
G. soja Sieb.& Zucc		40	G	G	51762		China, Japan, Russia, Korea, Taiwan	
G. max (L.) Merr.		40	G_1	G			Cultigen; worldwide	

Abbreviations: *N* nuclear, *C* Chloroplast, *PI* Plant introduction, *G* CSIRO (Commonwealth Scientific and Industrial Research Organization) number, *Aust* Australia, *Q* Queensland, *NSW* New South Wales, *NT* Northern Territory, *SA* South Australia, *V* Victoria, *WA* Western Australia, *T* Tasmanisa, *PNG* Papua New Guinea, *IL* Illinois

[a]Taiwan (Quemoy Is., Pescadores, Penghu Is., Paisha Is., Hsiaomen Is., Hsiyu Is., Chumei Is., Ghebay Is.,Tiachung), Japan (Ryukyu, Miyako Is., Ie Is., Kurima Is., Shimaji Is., Ishigaki Is.), Marianas (Tinian Is., Saipan Is.), Fiji, Tonga, Vanuatu, New Caledonia, Niue

cultivated phenotype displays a bush-type growth habit with a stout primary stem and sparse branches, bearing large seeds with variable seed coat colors, while the wild phenotype is a procumbent or climbing vine with a slender, many branched stem bearing small, coarse black seeds. The wild soybean also differs in the extent of hard seededness and pod dehiscence from the cultivated soybean, although genetic variations also exist in the latter for these traits.

Soybean is an annual plant. It exhibits taproot growth initially, followed later by the development of a large number of secondary roots. The roots establish a symbiotic relationship with the nitrogen fixing bacterium, *Bradyrhizobium japonicum*, through formation of root nodules. Soybean has four different types of leaves: the seed leaves (first pair of simple cotyledons) (Fig. 5.2a, b; *epigeal germination*), simple primary leaves (Fig. 5.2c), pinnately trifoliolate leaves (Fig. 5.2d), and the prophylls (a pair of 1 mm long simple leaves at the base of each lateral branch) [see Lersten and Carlson (2004) for detailed description of vegetative morphology].

Two loci are known to control stem termination (*Dt1* and *Dt2*; Woodworth 1933; Bernard 1972). With the determinate stem type (*dt1*), there is usually little growth in stem length after flowering, with blunt stem termination and a terminal raceme; whereas with the indeterminate stem type (*Dt1*), stem elongation and node production continue after flowering, producing a longer, more tapered main stem and branches. There is considerable variation in stem growth within each of these two types, with the time of flowering and of maturity having major effects on stem morphology. An intermediate phenotype conditioned by the *Dt2* genotype is called "semi-determinate" (Bernard 1972). Thompson et al. (1997) identified a third allele ($dt1 - t$) at the *Dt1* locus. It produces a phenotype for plant height that is similar to *Dt2* but with fewer main stems and nodes and larger terminal leaflets.

Soybean plants enter into reproductive stages following vegetative growth. Axillary buds develop into clusters of 2–35 flowers. From 20 to 80% of the flowers abscise (Carlson and Lersten 2004). Generally, the earliest and latest flowers produced abort most often. Soybean has a typical papilionaceous flower with a tubular calyx of five unequal sepals and a five-part corolla. The corolla consists of a standard posterior banner petal, two lateral wings and two anterior keel petals contacting with each other but not fused (Fig. 5.3a). The stamens are clustered around the stigma, ensuring self-pollination (Fig. 5.3b). The

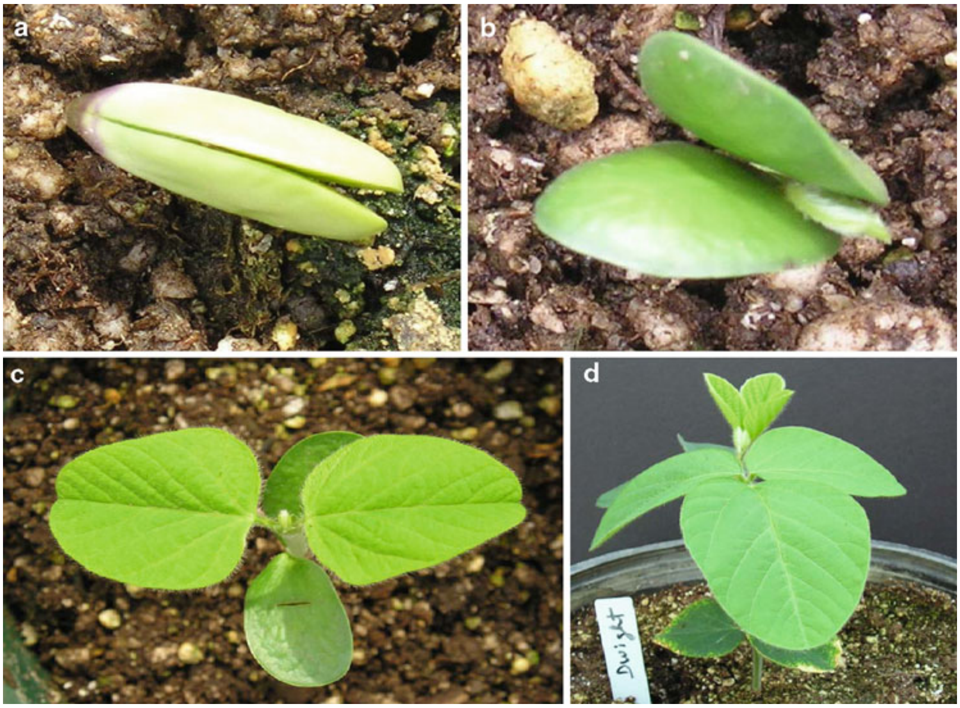


Fig. 5.2 Germination of soybean: (**a**) epigeal germination with two cotyledons leaves, (**b**) cotyledon leaves with emerging primary leaves, (**c**) simple primary leaves, (**d**) trifoliolate leaves [with permission from Chung and Singh (2008)]

gynoecium consists of an ovary, style, and stigma (Fig. 5.3c). As many as four ovules develop in the ovary. Nine stamens are arranged in two whorls; the outer and inner whorls contain five and four stamens, respectively. The two whorls of nine stamens align themselves into a single whorl on a staminal tube. The larger and older stamens alternate with the smaller and younger stamens in sequence around the developing gynoecium. The single free stamen (the tenth) is the last to appear (Fig. 5.3d). Soybean is highly self-pollinated with natural crossing usually below 1% because the stamens are elevated so that the anthers form a ring around the stigma. Thus, pollen is shed directly on the stigma surface, ensuring self-pollination (Carlson and Lersten 2004).

When the pollen grains are shed onto the stigma, they germinate, and the pollen tubes travel through style and enter into the filiform apparatus. The pollen tube tip bursts and releases two sperm nuclei. One sperm nucleus fuses with the egg nucleus and forms a zygote, while the second sperm unites with the two secondary nuclei, forming cotyledons. The seeds mature about 50 days after fertilization. They are devoid of endosperm and contain two large fleshy cotyledons, a plumule with two well-developed primary leaves enclosing one trifoliolate leaf primordium; a hypocotyl-radicle axis, a micropyle, and a hilum with a central fissure and a raphe (see Carlson and Lersten 2004).

The inflorescence of each node of the soybean plant may develop into one to more than 20 pods. A plant may produce up to 400 pods and over 1,000 seeds (Singh unpublished results). The soybean pod is similar to that of other legumes. A pod usually contains 1–3 seeds and rarely 4 seeds, except for plants that have the *na* allele that produces narrow leaflets and a much higher proportion of 4-seeded pods.

The stem of *G. max* is usually erect and produces axillary flowers (Fig. 5.4a), and wild perennial species are twining, climbing (Fig. 5.4b–d), or procumbent, and exhibit morphologically distinct traits. *Glycine cyrtoloba* and *G. curvata* contain curved pods (Fig. 5.4d); *G. microphylla* (Benth.) Tindale, *G. latifolia* (Benth.) Newell and Hymowitz, and *G. tabacina* (Labill.) Benth. carry adventitious roots, while *G. falcata* Benth. produces falcate pods (Fig. 5.4e). Wild perennial species show variation in flower color, from light purple (light pink; *G. falcata*) to dark purple, white, and blue.

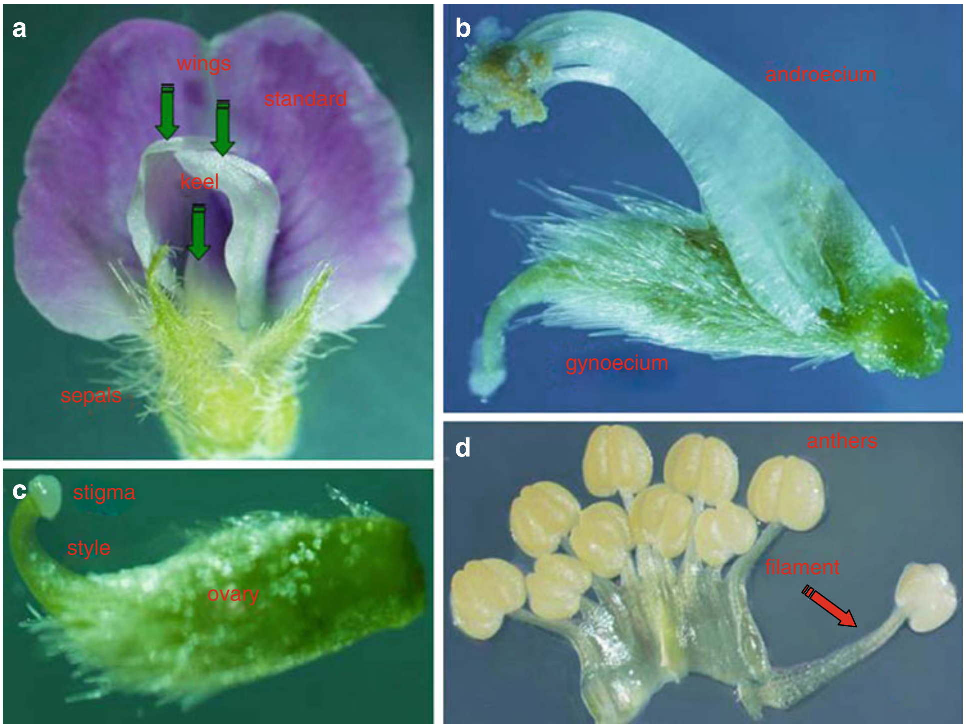


Fig. 5.3 Reproductive organs (identified) of soybean: (**a**) complete mature flower, (**b**) mature androecium, (**c**) a mature gynoecium with stigma, style, and ovary, (**d**) mature anthers with five anthers on longer filament (outer whorl), four anthers on shorter filament (inner whorl), and one free anther (*arrow*) always below the stigma [with permission from Singh et al. (2007b)]

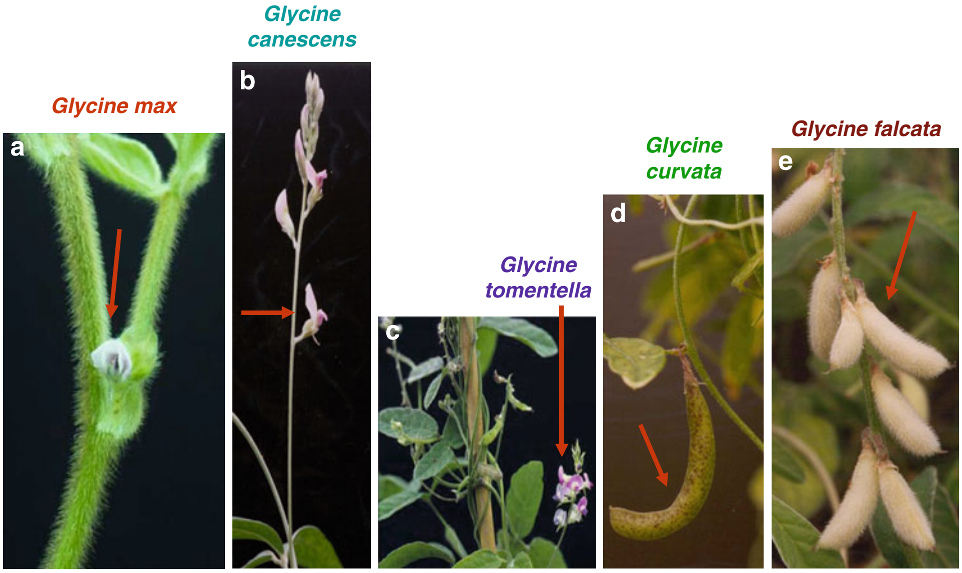


Fig. 5.4 Morphological diversity in *Glycine* species: (**a**). *Glycine max* with axillary flower, (**b**) *Glycine canescens* showing flowers on a long raceme, (**c**) *Glycine tomentella* with twinning stem and short raceme, (**d**) *Glycine curvata* with curved pod; a characteristic morphological trait, (**e**) *Glycine falcata* showing falcate pods on a raceme

5.3 Basic Chromosome Number of *Glycine*

The lowest chromosome numbers in *Glycine* ($2n = 38, 40$) are higher than those of related phaseoloid genera, among which $2n = 10$ and 11 predominates (Goldblatt 1981). Lackey (1980) summarized chromosome counts of 53 species out of a total of 84 for the tribe Phaseoleae and reported that "the unique chromosome number of *Glycine* is probably derived from diploid ancestors with base number 11, which have undergone aneuploid loss to base number 10 and subsequent polyploidy to give somatic counts of 40 or sometime 80." Such a hypothesis is not inconsistent with the chromosome numbers of the closest generic relatives suggested by the chloroplast phylogeny (Stefanovic et al. 2009): *Amphicarpaea*, $2n = 22$; Psoraleeae, $2n = 20, 22$; though *Teramnus* is $2n = 28$.

Zhu et al. (1995) discussed at great length the hypothetical ancestors of soybean based on comparisons of the sequence of the RFLP locus A-199a. *Neonotonia* ($2n = 22$) was the closest genus to *Glycine*, followed by *Amphicarpa* ($2n = 22$) and then *Teramnus* ($2n = 28$). By contrast, Lee and Hymowitz (2001) and Doyle et al. (2003) proposed that *Teramnus* is the closest relative of the *Glycine* on the basis of molecular taxonomy using a single chloroplast gene region; a more comprehensive study of chloroplast DNA sequence variation indicated Psoraleeae as the likely sister group to the chloroplast genome of *Glycine* and concluded that *Teramnus* and *Amphicarpaea* were also closely related (Stefanovic et al. 2009). Darlington and Wylie (1955) proposed $x = 10$ as the basic chromosome number of the genus *Glycine*. However, ancestral diploid *Glycine* species with $2n = 20$ have not been identified (Hadley and Hymowitz 1973; Lackey 1980; Kumar and Hymowitz 1989). Walling et al. (2006) reported that the 40 chromosomes of soybean have been "derived from at least two rounds of genome-wide duplication or polyploidy events." Thus, it has been assumed that the basic chromosome number of the genus *Glycine* (as with all phaseoloid legumes) is $x = 5$.

Genetic and genomic studies have corroborated some aspects of this hypothesis. The genetic linkage map of soybean shows evidence of two nested duplications (Lee et al. 1999), and duplications in the leghemoglobin gene family are consistent with whole-genome duplications (Lee and Verma 1984). More recently, consideration of divergence of duplicate (paralogous) gene pairs estimated from expressed sequence tags (ESTs) revealed the presence of two whole-genome duplications (Blanc and Wolfe 2004; Schlueter et al. 2004; Shoemaker et al. 2006). The older of these duplications, dated at around 50 Mya, is shared with *Medicago truncatula* (Pfeil et al. 2005; Cannon et al. 2006). *Medicago* is a member of one of the other major clades of papilionoid legumes, the Hologalegina, which also includes pea (*Pisum sativum*), clover (*Trifolium* species), broad bean (*Vicia faba*), and other crop plants (Lewis et al. 2005); it is the sister group to the millettioid legumes (Wojciechowski et al. 2004). However, the lowest chromosome numbers of Hologalegina are $2n = 14$ or 16 and not $2n = 10$ or 20. So it is unclear what the chromosome number of the common ancestor of Hologalegina and the phaseoloid legumes was. Clearly, however, there was evolution of chromosome number early in the evolution of the papilionoid legumes.

Although chromosome number and genetic/genomic data indicate that *Glycine* has undergone a second, more recent whole genome duplication, none of this information sheds light on whether that event involved the duplication of the genome of a single species (taxonomic autopolyploidy) or whether the origin of *Glycine* involved hybridization between two diploid species (taxonomic allopolyploidy). It had been hypothesized that a putative ancestor of the genus *Glycine* with $2n = 2x = 20$ likely arose in Southeast Asia and then underwent auto- or allopolyploidization (Darlington and Wylie 1955; Kumar and Hymowitz 1989; Singh and Hymowitz 1999; Lee and Hymowitz 2001; Singh et al. 2001). Initial molecular evolutionary studies concluded that *G. max* is an autotetraploid species (Gurley et al. 1979; Lee and Verma 1984; Skorupska et al. 1989), but other cytological (Hymowitz 1970; Xu et al. 2000a, b) and molecular research (e.g., Zhu et al. 1995) suggested that the species is a diploidized ancient (paleo) allotetraploid.

All of the *Glycine* species studied by Singh and Hymowitz (1985a) showed diploid-like meiosis, making modern *Glycine* a genetic allopolyploid with diverged homoeologous loci. It is assumed that plant species that begin their existence having multisomic inheritance characteristic of genetic autopolyploids eventually evolve to become chromosomally "diploidized" fixed heterozygotes with disomic segregation like plants that begin as genetic allopolyploids (Doyle et al. 2008). Thus, the current disomic genetic behavior of *Glycine* does not preclude an autopolyploid origin.

Gene phylogenies are capable of diagnosing an allopolyploid origin if the progenitors of an allopolyploid are not extinct, but a phylogenetic pattern in which the two homoeologous copies of a polyploid are sister to one another (rather than each being sister to a gene from a different diploid) is consistent either with autopolyploidy or allopolyploidy involving extinct diploid progenitors (Doyle and Egan 2010). The latter, unfortunately, is the pattern observed in *Glycine* (Doyle et al. 2003; Straub et al. 2006), leaving the question of auto- vs. allopolyploid origins unanswerable by phylogenetic data. A recent cytological analysis used fluorescence in situ hybridization (FISH) to show that two classes of centromeric heterochromatin repeats are localized on two nearly mutually exclusive sets of soybean chromosomes, suggestive of subgenomes derived from two progenitor genomes through allopolyploidy (Gill et al. 2009). Regardless of the mode of its origin, the *Glycine* is composed of blocks of duplicated, homoeologous sequences at the megabase scale (e.g., Innes et al. 2008; Cannon et al. 2009; Schmutz et al. 2010).

The timing of divergence of duplicated genes in the soybean genome provides an estimate for the age of the recent polyploid event that produced the current $2n = 40$ genome of "diploid" *Glycine*. This homoeolog divergence time lies between 10 and 15 million years (Schlueter et al. 2004, 2007; Innes et al. 2008). However, the divergence of homoeologs in an allopolyploid gives the divergence time of the diploid progenitors, and not the date of the polyploid event (Gaut and Doebley 1997; Doyle and Egan 2010), so the 10–15 Mya date for *Glycine* is the maximum age. The minimum age of the polyploid event is approximately 5 Mya, the age estimated for the earliest speciation events within *Glycine*, which divided the soybean lineage from the Australian perennial species (Innes et al. 2008). This estimate is based on the parsimonious assumption that all of the $2n = 40$ species in the genus are derived from the same polyploidy event.

5.4 Geographical Distribution of *Glycine*

Table 5.3 shows the current information on the genus *Glycine*. The cultivated soybean, *G. max*, has a worldwide distribution. Morphologically, the species is variable in characteristics that have been the targets of human selection, such as seed coat color and pattern.

G. soja is distributed throughout China and in adjacent areas of the lowlands of the Russian Far East region, Korea, and Japan (Hymowitz et al. 1998; Shimamoto et al. 1998; Tozuka et al. 1998; Zhuang 1999; Chung and Singh 2008), at the latitudes between approximately 24° and 53°N and the longitudes between approximately 97° and 143°E. The annual wild soybeans are typical ruderal plants and are usually found in semi-disturbed areas. This is particularly true of the weedy "*G. gracilis*" (see below, which grows mainly as a twining vine around relatively tall herbs or bushes). When there are no tall plants to climb, the wild soybeans show prostrate or bush forms (Ohara and Shimamoto 1994, 2002). In China, *G. soja* has a particularly wide distribution and is found in nearly all provinces, except for Hainan, Qinghai, and Xinjiang Provinces, occurring on waste land, mountain slopes, in farmer's fields, and along field margins, hedgerows, roadsides, riverbanks, lakesides, and in marshland, at altitudes from 5 to 2,650 m above sea level. The ruderal nature of *G. soja* makes it difficult, if not impossible, to determine the original native range of the species.

G. gracilis Skvortz. is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. Once considered an intermediate in the speciation of *G. max* from *G. soja* (Fukuda 1933), it is more likely an early generation hybrid between the wild *G. soja* and the cultigen, *G. max* (Hymowitz 1970), usually being found in areas where the cultivated soybean and its wild ancestor have a sympatric distribution. It was mostly reported in the northeast part of China (Hymowitz 1970). However, herbarium specimens collected from a wider geographic range show intermediate characteristics between *G. max* and *G. soja*. Therefore, more detailed surveys may reveal a more extensive occurrence of this weedy soybean in China as well as adjacent areas (Korea and Japan) in which *G. max* and *G. soja* coexist. Nevertheless, the weedy soybean was never reported in other parts of the world such as the Americas and Europe where only the cultivated soybean was grown. Information on the geographic distribution of close relatives of the cultivated soybean is extremely important for the estimation of the effect of transgene escape from the cultivated soybean to its wild relatives through gene

flow. This is because spatial contact or sympatric distribution is the prerequisite for regular gene exchange between two taxa, provided that a certain frequency of cross-pollination occurs.

All of the diploid species of subgenus *Glycine* are confined to Australia with the exception of *G. tomentella* D3, which also occurs in eastern Papua New Guinea (Hymowitz and Singh 1987; Shimamoto 1999, Doyle et al. 2003). Of the eight tetraploid species of the subgenus, five also occur outside of Australia in islands of the South Pacific, and as far north as Taiwan and the Ryukyu chain (Hymowitz and Singh 1987; Zhuang 1999; Doyle et al. 2002; Singh et al. 2007a, b; Chung and Singh 2008). Morphologically, most of these perennial species are twining vines somewhat reminiscent of *G. soja*. In addition to producing showy, chasmogamous flowers capable of outcrossing, many, if not all, species produce selfing, cleistogamous flowers. *G. falcata* and *G. albicans* are geocarpic.

5.5 Conservation Initiatives

With the rapid development of the global economy and continued increase in world population, natural environments face serious deterioration and change, which has led to the extinction or severe endangerment of many plant species including important crops and their wild relatives. At the same time, humans depend more and more on the wild relatives to enhance crop productivity in breeding by transferring genes from the wild gene pool. Effective conservation and exploitation of these wild relatives and their genetic diversity become essential factors in guaranteeing global food security and sustainable crop production. A great number of wild soybean populations has unfortunately become extinct or has been significantly reduced in recent decades due to the loss of a significant portion of their natural habitats at many locations in China. It is, therefore, vitally important to conserve wild soybean genetic resources.

For effective conservation of genetic diversity presented in wild soybean natural populations, either through in situ or ex situ approach, it is essential to have an appropriate sampling strategy to capture possibly high genetic diversity with a manageable amount of samples. For sampling in a wild soybean population, after an appropriate number of individuals to be collected are determined, the spatial intervals among individuals become another essential parameter for achieving a successful sampling strategy. Many studies have been done to evaluate the genetic diversity within accessions of wild soybean (Yu and Kiang 1993; Maughan et al. 1995; Choi et al. 1999; Dong et al. 2001; Kuroda et al. 2006; Li and Nelson 2001, 2002; Maughan et al. 1995; Wang and Takahata 2007; Yu et al. 1993; Xu et al. 2002; Xu and Gai 2003; Cho et al. 2006). However, little attention has been given to the fine scale genetic structure within natural populations of wild soybean, which is crucial for its ex situ conservation. An extensive wild soybean exploration in South Korean peninsula has produced over 4,000 accessions (Chung personal communication). Because the subgenus *Glycine* is the tertiary (GP-3) or quaternary (GP-4) gene pool for the cultivated soybean and harbors desirable agronomic traits, such as drought tolerance and disease and insect resistance, the group has been collected extensively. More than 2,000 accessions of the subgenus *Glycine* germplasm were collected, which provided the foundation for its biosystematic and molecular phylogenetic studies (Doyle et al. 2003), and a methodology has been developed to produce fertile plants between soybean (GP-1) and *G. tomentella* ($2n = 78$; GP-3) (Singh 2007). This method unlocks new, useful, economically important genes, not present in the soybean gene pool, from *G. tomentella* (Singh unpublished results).

5.6 Genetic Diversity in *Glycine*

The key factor for the effective conservation of genetic diversity is to accurately measure the genetic diversity of the targeted plant species. It will become possible to compare the level of genetic diversity among populations or species only when estimation of the total genetic diversity of the populations and species is correct and objective. During the past two decades, literature on genomic relationships in *Glycine* has been dominated by cytogenetics and molecular studies, including nuclear [seed protein electrophoresis, isozyme variation, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), simple sequence repeat (SSR),

sequences variation in the gene such as internal transcribed region (ITS) region of rDNA], extra-nuclear (chloroplast and mitochondrial DNA variation), and genomic in situ hybridization (GISH) by multicolor FISH. Molecular techniques are extremely powerful tools, especially for determining species relationships where production of interspecific or intergeneric hybrids is not feasible by conventional methods (Singh 2003).

The level of genetic diversity and geographic differentiation in Chinese cultivated soybean have been extensively studied using the coefficient of parentage (Cui et al. 2000a, b), morphological traits (Dong et al. 2004), RFLPs of chloroplast and mitochondrial DNA (Shimamoto et al. 2000), RAPD (Li et al. 2001) and simple sequence repeat (SSR) markers (Li et al. 2008; Wang et al. 2008), showing a clear geographic effect on genetic structure. Comparisons between the diversity of different samples of Asian soybean landraces and that of North American cultivars have demonstrated a lower level of diversity in the American pools than in the Asian pools, using either phenotypic characterization (Cui et al. 2000a, 2001) or the coefficient of parentage (Cui et al. 2000b). This reduced diversity was confirmed using sequence analyses to show successive genetic bottlenecks between wild and cultivated soybeans and between Asian landraces and North American cultivars (Hyten et al. 2006). Genetic diversity in soybean is covered in great detail by Carter et al. (2004).

Many studies have been conducted to evaluate the genetic diversity within accessions of wild soybean species (Kuroda et al. 2006; Lee et al. 2008; Li and Nelson 2001, 2002; Shimamoto et al. 1998; Wang and Takahata 2007; Xu et al. 2002). During the long-term evolutionary process, the wild soybean (*G. soja*) has accumulated tremendously rich genetic diversity by natural selection and its adaptation to various ecologic conditions and the continued changes of the environments. This adaptive evolutionary process has eventually led to the formation of diversification patterns of the wild soybean (Zhuang 1999). Soybean and its wild annual relative *G. soja* contain a great deal of diversity (Carter et al. 2004). This includes diversity for many obvious morphological traits like flower, pubescence, seed and hilum color, disease, and insect resistance traits, physiological and biochemical traits, as well as content of protein, oil, and carbohydrates and their constituents (Boerma and Specht 2004). *Glycine soja* is a source of genetic variation to improve soybeans. To improve the efficiency evaluation of conserved germplasm, a core or mini-core collection approach that maximizes allelic diversity in a proportion of the whole collection has frequently been advocated. In addition to being essential for elucidation of the origins and evolution of cultivated soybean, knowledge gained in annual wild soybean may provide useful genetic information for more efficient improvement of soybean cultivars.

The genomic relationships among most of the diploid ($2n = 40$) perennial species in the subgenus *Glycine* have been reviewed by Chung and Singh (2008). These relationships were established using cytogenetic methods, biochemical techniques, and molecular tools. Thus, species with the same genome designation are expected to be able to be crossed to produce viable, vigorous, and fertile F_1 plants (Table 5.3). For *Glycine* species with dissimilar genome designations, the crossability is extremely low, the pods and/or seeds may abort, and any seedlings that develop are weak and sterile (Singh and Hymowitz 1988; Singh et al. 1992a; Kollipara et al. 1993). A much more detailed discussion of the species and species relationships in the subgenus *Glycine* is presented by Chung and Singh (2008). The polyploid and aneuploid members of the perennial species have been studied less and appear to have more complex relationships. Allopolyploidization has played a key role in the recent diversification of subgenus *Glycine* and, as noted above, may have been involved in the origin of the genus as a whole.

5.7 National and International Germplasm Collection

The largest collection of soybean germplasm is at the National Crop Gene Bank in Beijing, China, with 25,034 accessions of *G. max* and 6,172 accessions of wild soybean (*G. soja*) (*http://icgr.caas.net.cn/cgris introduction.html*: accessed 15 March 2010). The USDA-ARS, Urbana, IL, USA collection (*http://www.ars-grin.gov/cgi-bin/npgs/html/site_holding.pl?SOY*: accessed 15 March 2010) holds 1,181 accessions of wild soybean and 18,932 accessions of *G. max*.

More than 70 additional countries maintain more than 170,000 *G. max* accessions (Carter et al. 2004)

and fewer than 10,000 *G. soja* accessions. Many remote regions of Southeast Asia including Vietnam, Laos, Cambodia, Myanmar, and the northeastern and North Himalayan regions have not been explored as yet for landraces of soybean and wild soybean. The USDA Soybean Germplasm Collection, Urbana, Illinois, maintains 996 accessions of the 19 wild perennial species (*http://www.ars-grin.gov/cgi-bin/npgs/html/site_holding.pl?SOY*), and CSIRO maintains the largest collection of perennial *Glycine* species.

5.8 Phylogenetic Studies in *Glycine*

5.8.1 Taxonomy, Crossability, and Classical Studies

The name *Glycine* was originally introduced by Linnaeus in his first edition of *Genera Plantaram* (Linnaeus 1737). This new genus was based on *Apios* of Boerhaave (Linnaeus 1753). *Glycine* is derived from the Greek *glykus* (sweet). The name probably refers to the sweetness of the edible tubers produced by *G. apios* L. (Henderson 1881), now known as *Apios americana* Medik. Linnaeus in his 1753 publication *Species Plantarum* listed eight *Glycine* species. All these were subsequently moved to other genera; thus the Greek word *glykys* currently does not refer to any *Glycine* species (Hymowitz and Singh 1987). Since the time of Linnaeus, scholars have discussed the correct nomenclature for the cultivated soybean. The combination *G. max* as proposed by Merrill in 1917 is now generally accepted as the valid designation of the cultivated soybean. The genus *Glycine* has had many species added to it and subsequently removed over the years, including the removal of the original lectotype (Bentham 1864, 1865; Hitchcock and Green 1947; Hermann 1962; Verdcourt 1970; Lackey 1977a, b; Hymowitz and Singh 1987; Hymowitz 2004; Orf 2010).

There has been no adequate comprehensive study of biosystematic and evolutionary relationships of all species in the genus *Glycine*. However, the annual (subgenus *Soja*) and perennial (subgenus *Glycine*) soybean species are significantly distantly related (Doyle et al. 2003), having diverged from a common ancestor around 5 Mya (Innes et al. 2008). Early attempts to hybridize species between the subgenus *Soja* and subgenus *Glycine* were unsuccessful. In these studies, the pods resulted from interspecific hybridization that was eventually aborted and abscised although pod development could be initiated (Ladizinsky et al. 1979; Hood and Allen 1980). Later, the intersubgeneric F_1 hybrids of *G. max* × *G. clandestina*, *G. max* × *G. tomentella,* and *G. max* × *G. canescens* were obtained in vitro either through embryo rescue (Newell and Hymowitz 1982; Singh and Hymowitz 1985d; Singh et al. 1987a) or using transplanted endosperm as a nurse layer (Broué et al. 1982). In general, it is concluded that the cultivated soybean could only hybridize with members of the subgenus *Glycine* imperfectly and under extreme technical assistance. In all cases, the progeny of such intersubgeneric hybrids were completely sterile and obtained with great difficulty. Research also demonstrated that the cultivated soybean does not hybridize with any of the wild relatives in other genera of the tribe (Hymowitz and Singh 1987).

Crossability rate is an excellent indirect measure for estimating the degree of genomic relationship between parental species. Interspecific crosses involving parental species with similar genomes usually set normal pods, and F_1 seeds and the hybrids are fertile, while in crosses between genomically dissimilar species, seed abortion is common, and hybrids, if obtained, are sterile (Newell and Hymowitz 1983; Grant et al. 1984a, b, 1986; Singh and Hymowitz 1985b, c; Singh et al. 1987b, 1989, 1992a, 1998b; Kollipara et al. 1993). Normal pod set and fertile F_1 hybrids are expected from crosses between morphologically and genomically similar species. However, this is not always true. For example, *G. cyrtoloba* and *G. curvata* carry curved pods, and their morphological features are nearly identical (Tindale 1984, 1986a).

In contrast, the cultivated soybean can be easily hybridized with *G. soja* and *G. gracilis* and produce fertile offspring. Interspecific hybridization between *G. max* and *G. soja* (Hadley and Hymowitz 1973; Ahmad et al. 1977, 1983, 1984; Broich 1978) and between *G. max* and *G. gracilis* (Karasawa 1952; Singh and Hymowitz 1989) was performed and fertile interspecific hybrids were obtained without much difficulty. Hybrids between the diploid annual *Glycine* species usually show normal meiosis and are fertile (Zhuang 1999). This strongly supports the viewpoint

that the cultivated soybean and the two annual wild relatives share a common primary gene pool. As a matter of fact, *G. max* and *G. gracilis* are extensively used in soybean breeding programs to transfer elite germplasm through sexual interspecific hybridization. This indicates a significantly close biosystematic relationship among the three species. Molecular studies of genetic diversification and variation patterns of *G. max*, *G. soja*, and *G. gracilis* also confirmed a particularly close evolutionary relationship between the three species (Hui et al. 1996; Powell et al. 1996; Hui 1997; Wu et al. 2001).

Based on classical taxonomy, *G. soja* and *G. max* are different species (Hermann 1962). Both species carry $2n = 40$ chromosomes, hybridize readily, produce viable, vigorous, and fertile hybrids, and some lines differ by a reciprocal translocations (Karasawa 1936; Palmer et al. 1987; Singh and Hymowitz 1988) or by paracentric inversions (Ahmad et al. 1977, 1979). Therefore, *G. soja* and *G. max* have now been assigned genome symbols G and G_1, respectively (Singh et al. 2007b). Putievsky and Broué (1979) laid the foundation of genomic relationships in the genus *Glycine*. They produced 19 intraspecific and 30 interspecific F_1 hybrids among *G. canescens*, *G. clandestina*, *G. tomentella* ($2n = 78, 80$), *G. falcata*, and *G. tabacina* ($2n = 40, 80$). In the genus *Glycine*, all F_1 hybrids from crosses among A-(*G. canescens*, *G. argyrea*, *G. clandestina*, and *G. syndetika*) and B-(*G. microphylla*, *G. latifolia*, and *G. tabacina*) genome species displayed 20 bivalents at metaphase-I in the majority of the sporocytes (Broué et al. 1979; Putievsky and Broué 1979; Newell and Hymowitz 1983; Grant et al. 1984a, b; Singh and Hymowitz 1985b, c; Singh et al. 1988, 1992a, 2007b).

Classical taxonomy has played a major role in the identification and nomenclature of new species in the subgenus *Glycine* (Table 5.3). *Glycine clandestina* ($2n = 40$) has been observed to be a morphologically highly variable species (Hermann 1962). Newell and Hymowitz (1980) revised the subgenus *Glycine* by proposing a new species, *G. latifolia* Benth. (Newell and Hymowitz). Short pod *G. clandestina* (Singh and Hymowitz 1985b) was removed and named *G. microphylla* (Benth.) Tind. (Tindale 1986b) and curved pod *G. clandestina* was classified as *G. cyrtoloba* (Tindale 1984) and *G. curvata* (Tindale 1986a). Costanza and Hymowitz (1987) observed the presence of adventitious roots in B-genome species (Table 5.3) that includes *G. microphylla*, *G. latifolia*, and *G. tabacina*. This morphologically distinguishing trait is absent in other *Glycine* species. Tindale and Craven (1988) described three new species (*G. albicans* Tindale and Craven, *G. lactovirens* Tind. and Craven, and *G. hirticaulis* Tind. and Craven). *Glycine hirticaulis* contains accessions with $2n = 40$ and 80 chromosomes. Newly described species have restricted geographical habitats in Australia, and they have proven difficult to maintain under greenhouse conditions.

5.8.1.1 *G. tomentella*

Diploid ($2n = 40$) *G. tomentella* accessions have been separated into different groups [D1, D2, D3 (A, B, C), D4, D5, D6, and D7] based on isozyme similarities (Doyle and Brown 1985; Doyle et al. 1986). Later, accessions of the D6 group from Western Australia were classified as *G. arenaria* Tind. (Tindale 1986b). Singh and Hymowitz (1985b) produced and studied meiotic chromosome pairing between D4 group *G. tomentella* (PI 441000) and *G. clandestina* ($2n = 40$; PI 440948; A_1-genome). The PI 441000 accession (D4) contains long pods and narrow leaves, and its meiotic pairing suggests that it is closer to A-genome species (Singh et al. 1992a; Kollipara et al. 1995). Pfeil et al. (2006) removed PI 441000 and other D4 accessions from *G. tomentella* and named these *G. syndetika* B.E. Pfeil and Craven. Singh et al. (2007b) assigned to it a genome symbol A_6. Currently, we have 26 classified wild perennial *Glycine* species because of extensive plant exploration and taxonomic and molecular studies (Table 5.3).

Of the 26 wild perennial *Glycine* species, *G. tomentella* is a unique species because it consists of four cytotypes ($2n = 38, 40, 78, 80$). However, *G. tomentella* is not a single species in a biological or genetic sense, because the diploid "isozyme races" noted above are reproductively isolated from one another. It is thus a species complex, whose morphology defies simple separation into separate taxa because the various allopolyploids are fixed hybrids, combining features of their diploid progenitors (see Doyle et al. 2002 for detailed discussion of this complex). Aneudiploid ($2n = 38$) *G. tomentella* is distributed in a restricted region of Queensland, Australia. The diploid ($2n = 40$) cytotype is distributed widely in Australia (Queensland, Northern

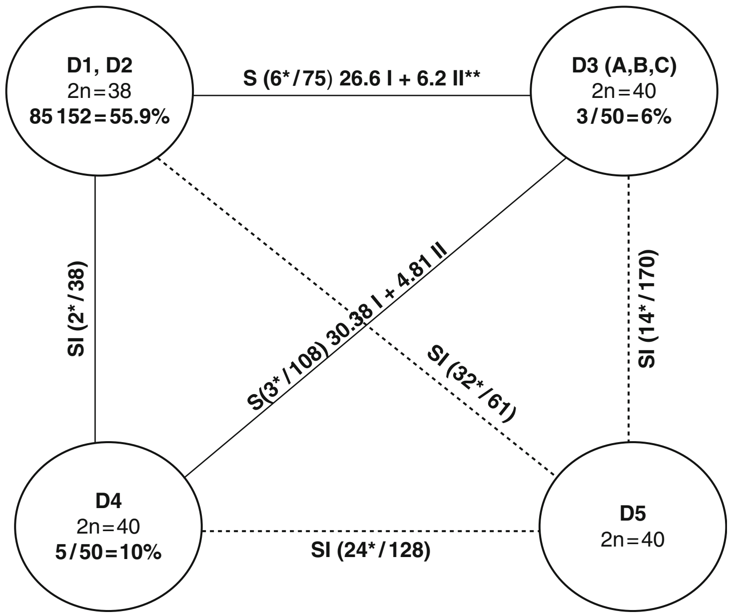


Fig. 5.5 Summary of genomic relationships among five groups of aneudiploid ($2n = 38$; D1 and D2) and diploid [$2n = 40$; D3 (A, B, C), D4, and D5] *G. tomentella* based on crossability rate and meiotic chromosome pairing in F_1 hybrids. The within-group crossability rate (%) is shown inside the *circle*. The between-group crossability rates (number of pod set/total number of flowers pollinated) are shown in the *parenthesis*. *Asterisk* number of aborted pods; *double asterisk* Singh et al. (1988); *S* sterile, *SI* seed inviability [with permission from Kollipara et al. (1993)]

Territory, Western Australia) and Papua New Guinea. Isozyme banding patterns grouped the aneudiploids into two isozyme groups (D1 and D2), and the diploids form six isozyme (D3A, D3B, D3C, D4, D5, D6) groups (Doyle and Brown 1985). Cytogenetics revealed that D1 and D2 isozyme groups carry a similar genome and are distinct from other isozyme groups, and Singh et al. (1988) assigned to it the E-genome symbol. (Fig. 5.5; Grant et al. 1984b; Singh et al. 1988). The histone H3-D gene sequence also grouped D4 isozyme accessions with A-genome species (Brown et al. 2002), and the D4 isozyme group *G. tomentella* was classified as *G. syndetika* (Pfeil et al. 2006); the A_6 genome symbol was assigned to PI 441000 (Singh and Chung 2007; Singh et al. 2007b). No viable hybrid plants were produced in crosses between accessions of the D5 and D1, D2, D3, and D4 isozyme groups (Fig. 5.5).

The genus *Glycine* offers a good model for studying evolution in natural allopolyploids. The primarily Australian perennial subgenus *Glycine* includes a large, recently formed allopolyploid complex comprising several diploid genomes that have formed polyploids in various combinations, often recurrently (Doyle et al. 2004). This complex has long been recognized as polytypic, with polyploids classified under three distinct species epithets: *G. tabacina*, *G. pescadrensis* Hayata (formerly known as *G. tabacina* AAB9B9), and *G. tomentella* (reviewed in Doyle et al. 2004).

The progenitor of the wild perennial species of the subgenus *Glycine* radiated out into several morphotypes depending on the growing conditions in the Australian continent. These species were never domesticated and remained wild perennials. In contrast, the pathway of migration from a common progenitor to East Asia is assumed as: wild perennial ($2n = 4x = 40$; unknown or extinct), wild annual ($2n = 4x = 40$; *G. soja*), soybean ($2n = 4x = 40$; *G. max*); diagrammatically shown in Fig. 5.6. All species of the genus *Glycine* with $2n = 38$, 40, 78, and 80 chromosomes exhibit diploid-like meiosis and are inbreeders (Singh and Hymowitz 1985a, b, c).

The comprehension of genomic relationships among species is important to systematists, evolutionary biologists, cytogeneticists, molecular biologists, and plant breeders. The taxonomic nomenclature of species and their evolutionary relationships can be refined by cytogenetic evidence such as chromosome

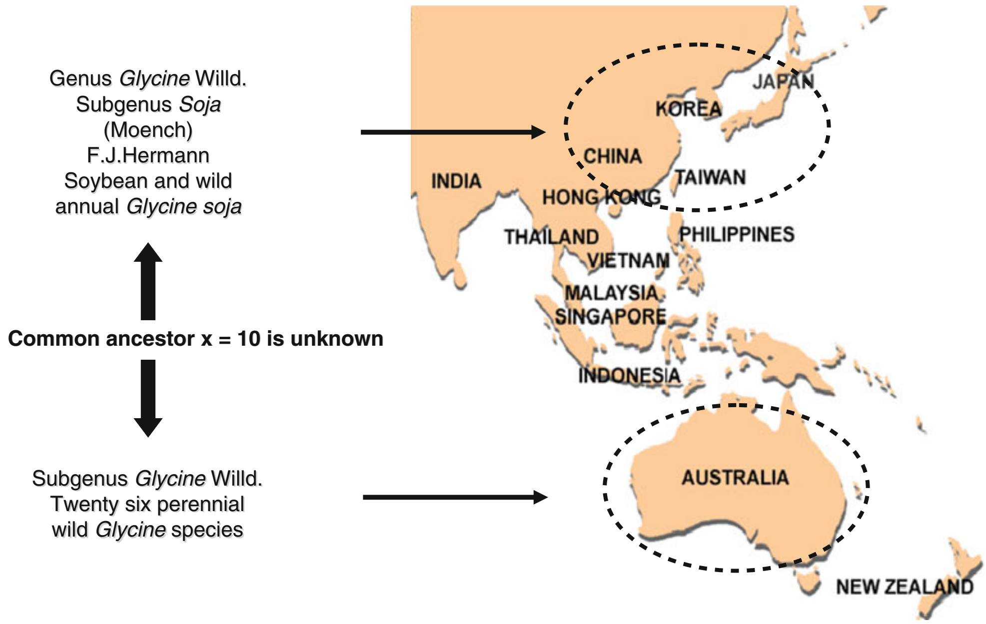


Fig. 5.6 A geographical map showing the home of *Glycine*; the common progenitor $2n = 2x = 20$ of *G. soja* and soybean (both annual) and 26 wild species (perennial) is unknown. It may be extinct or not yet identified. Soybean is domesticated in East Asia from *G. soja* (*circle*), and 26 wild perennial *Glycine* species, indigenous, (*circle*), were not domesticated in Australia [with permission from Singh et al. (2007b)]

morphology, crossability, hybrid viability, meiotic chromosome pairing, and molecular approaches (isozymes, nuclear, chloroplast, mitochondrial DNA markers, and FISH). Thus, phylogenetic relationships among species can be established more precisely by a multidisciplinary approach rather than through reliance on a single technique (Singh 2003).

The genomes of the diploid *Glycine* species ($2n = 40$) have been assigned capital letter symbols according to the degree of chromosome homology between species in F_1 hybrids (Kihara and Lilienfeld 1932). Similar letter symbols are designated for species with interspecific F_1 hybrids that show normal chromosome pairing. Placing a subscript after the letter indicates minor chromosome differentiation, such as inversions or translocations. Highly differentiated species are designated by different letter symbols because their hybrids exhibit highly irregular chromosome pairing and hybrids are completely sterile. Singh and Hymowitz (1985b) conceived the idea of assigning genome symbols to *Glycine* species based on cytogenetic results. Molecular methods helped to assign genome symbols to those species for which cytogenetic information was not obtained (Singh et al. 1988, 1992a; Kollipara et al. 1995, 1997; Brown et al. 2002; Doyle et al. 2002; Table 5.3).

5.8.1.2 Origin of Polyploid Complexes of *Glycine tabacina* and *G. tomentella*

This complex topic has been reviewed by Doyle et al. (2004). Although these complexes are discussed separately, due to their nomenclatural history as separate species, there is a single large complex of approximately eight polyploid species that unite around eight different diploid genomes in various combinations.

Glycine tabacina ($2n = 80$)

Diploid *G. tabacina* is one of three formally recognized diploid species of the taxonomically complex B-genome, all of which are indigenous to Australia. Tetraploid ($2n = 80$) *G. tabacina* is found sympatrically with B-genome diploids in Australia and is also distributed in the islands of the South Pacific (New Caledonia, Vanuatu, Fiji, Tonga, Niue) and West-central Pacific (Taiwan, Ryuku, Marianas) (Singh et al. 1992b). Morphological observations (Costanza and Hymowitz 1987), cytogenetic investigation (Singh et al. 1987b, 1992b), and molecular studies (Doyle et al. 1990a, b, c, 1999a) identified two distinct species from what had been called tetraploid *G. tabacina*.

Both are allopolyploids with multiple origins and share only one of their two genomes. What is now recognized as *G. tabacina* contains adventitious roots (designated as WAR), while the other species, *G. pescadrensis*, formally reinstated from synonymy with *G. tabacina* (Pfeil et al. 2006), has long and narrow leaves (like A-genome species) and has no adventitious roots (designated as NAR). Singh et al. (1992a, b) proposed, based on cytogenetics, that one genome of *G. pescadrensis* (NAR) was most likely contributed by an A-genome species (e.g., *G. canescens*, *G. clandestina*, *G. argyrea*, *G. syndetika*); later molecular studies identified *G. syndetika* as the direct progenitor (Doyle et al. 2000). The second homoeologous genome of *G. pescadrensis* was contributed by a diploid species sister to the core species of the B-genome, *G. stenophita* (Doyle et al. 2000). The *G. stenophita* genome is also found in polyploid *G. tabacina* (WAR), the other portion of whose allopolyploid genome was contributed by core B-genome species (*G. latifolia*, *G. microphylla*, *G. tabacina, G. stenophita*) through multiple origins (Doyle et al. 1999a, b, 2000). Doyle et al. (1999b) suggested that, based on sequencing of histone H3-D locus, the multiple origins with gene exchange among lineage increase the genetic base of a polyploid and help better colonization of polyploid *G. tabacina* relative to its diploid progenitors. Hybridization is unlikely in a highly self-inbreeding species in nature; however, F_1 hybrids among B-genome species are completely fertile (Newell and Hymowitz 1983; Putievsky and Broué 1979; Singh and Hymowitz 1985c).

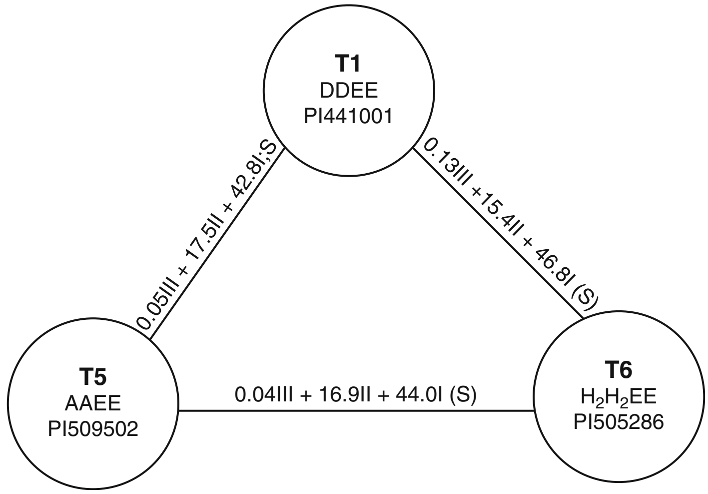
G. tomentella (2*n* = 78, 80)

Relationships among the various diploid and polyploid taxa that comprise *G. tomentella* have been hypothesized from crossing studies, isozyme data, and repeat length variation for the 5S nuclear ribosomal gene loci. However, several key questions have persisted, which have been addressed by detailed phylogenetic evidence from homoeologous nuclear genes. The histone H3-D locus is single copy in diploid *Glycine* species and has been used to elucidate relationships among diploid races of *G. tomentella* (Brown et al. 2002), providing a framework for testing genome origins in the polyploid complex. For all six *G. tomentella* polyploid races (T1-T6), alleles at two homoeologous histone H3-D loci were isolated and analyzed phylogenetically with alleles from diploid *Glycine* species, permitting the identification of all of the homoeologous genomes of the complex (Doyle et al. 2002). Allele networks were constructed to subdivide groups of homoeologous alleles further and two-locus genotypes were constructed using these allele classes. Results suggest that some races have more than one origin and that interfertility within races has led to lineage recombination. Most alleles in polyploids are identical or closely related to alleles in diploids, suggesting recency of polyploid origins and spread beyond Australia. These features parallel the other component of the *Glycine* subgenus *Glycine* polyploid complex, *G. tabacina* and *G. pescadrensis*, the latter of which shares the *G. syndetika* genome with *G. dolichocarpa* (aka *G. tomentella* T2).

Diploid-like meiosis, isozyme banding patterns among the accessions and meiotic pairing in intraspecific and interspecific F_1 hybrids, wide geographical distribution, and aggressive and vigorous growth habit suggest that 78 and 80-chromosome tomentellas are of allopolyploid origin and are polyploid complexes. Genomic complexes within species can be determined by obtaining intraspecific hybrids involving parental accessions of diverse morphology, cytology, and geographic origins. Meiotic pairing and molecular results in intraspecific plants of 78-chromosome tomentellas have revealed three complexes (designated based on isozyme variation; T1, T5, and T6; Doyle and Brown 1985). Hybrids within groups showed normal chromosome pairing. All hybrids between genomic complexes showed one common genome (EE-genome; 38 chromosome *G. tomentella*), and this was verified by molecular methods (Kollipara et al. 1994). This suggests that some complexes have one genome in common and differ for the second genome. T1 group aneutetraploid tomentella predominates and is distributed in Queensland, with one accession in Papua New Guinea. T5 group is found in New South Wales, while T6 group is found in Western Australia. Thus, these isozyme groups are geographically isolated and may have originated independently with an aneudiploid ($2n = 38$; E-genome) as the common genome donor (Fig. 5.7).

Based on isozyme banding patterns, Doyle and Brown (1985) separated 80-chromosome *G. tomentella* accessions into the T2, T3, and T4 groups. They did not examine accessions from Timor Island of Indonesia, and Kollipara et al. (1994) assigned these

Fig. 5.7 Summary of the genomic relationships based on an average chromosome pairing among three isozyme groups (T1, T5, and T6) in 78-chromosome *G. tomentella*. *S* sterile [with permission from Kollipara et al. (1994)]



accessions to the T7 group. Cytogenetics, total seed protein profiles, protease inhibitor activity band profiles, immunostained banding patterns, and RFLP analysis clearly identified four distinct groups (T2, T3, T4, and T7) in 80-chromosome *G. tomentella* (Kollipara et al. 1994). Chromosome pairing results suggest one common genome in the T2, T3, T4, and T7 groups. A way to ascertain the ancestors of 78-and 80-chromosome tomentellas is to synthesize amphidiploids from their putative parental species (Singh et al. 1987a, b, 1989). Aneuallotetraploid (DDEE, AAEE; $2n = 78$) and allotetraploid (AADD; $2n = 80$) were produced by somatic chromosome doubling of $2n = 39$ and $2n = 40$ F_1 hybrids. Synthesized amphidiploids were hybridized with accessions of tomentellas of T1, T5 ($2n = 78$) and T2 ($2n = 80$) isozyme groups. Meiotic pairing was normal and fertile (Singh et al. 1989), and molecular results verified the cytogenetic results (Kollipara et al. 1994). Rauscher et al. (2004) reported that the genome donors of T2 *G. tomentella* are diploid *G. tomentella* of D3 and D4 isozyme groups. The D4 (PI 441000) isozyme showed genomic affinity with *clandestina* (PI 505161) (Singh et al. 1987). Pfeil et al. (2006) taxonomically classified PI 441000 as *G. syndetika*, and Singh et al. (2007) assigned it the genome symbol A_6 (Table 5.3). Cytogenetics and molecular studies of Kollipara et al. (1994) supported *G. canescens* or any A-genome species, such as *G. syndetika,* as possible genome donor to T2 tomentella; histone H3-D phylogenies narrowed this to *G. syndetika* (Doyle et al. 2002), which, as noted above, was also a genome donor to allopolyploid *G. pescadrensis*, and thus connects the "*G. tabacina*" and "*G. tomentella*" complexes. T2 is now known to correspond to *G. dolichocarpa*, identified from Taiwan. T7 combines the same genomes as T3 (D3 × D5A) but involves different genotypes based on histone H3-D sequencing (Doyle et al. 2002). Histone results were subsequently corroborated with phylogenies from the nuclear ribosomal internal transcribed spacer region (ITS; Rauscher et al. 2004).

5.8.2 Cytological Studies

The degree of chromosome pairing in interspecific hybrids provides an important cytogenetic context for interpreting phylogenetic relationships among diploid species, enhances our understanding of the evolution of the genus, and provides information about the ancestral species. Generally, species with similar genomes exhibit complete or almost complete chromosome pairing (intragenomic chromosome pairing) in their hybrids.

The extent of chromosome association in the hybrids of genomically dissimilar species elucidates structural homology in the parental chromosomes and hence furnishes evidence regarding the progenitor species (Singh and Hymowitz 1985b; Singh 2003). Usually, the F_1 generated from genomically unlike parents (different biological species) are germinated through in vitro techniques. Hybrid seed inviability,

seedling lethality, and vegetative lethality are common occurrence in intergenomic crosses (Newell and Hymowitz 1983; Singh et al. 1988, 1992a). In general, such hybrids are weak, slow in vegetative and reproductive growth, and sterile. Variable (semihomologous-homoeologous) and minimal chromosome pairing are common in intergenomic F_1 hybrids. Aneudiploid ($2n = 38$) *G. tomentella* is morphologically similar to 40, 78, and 80 chromosome tomentellas. In contrast, limited chromosome pairing was observed between 38 and 40-chromsome *G. tomentella* (D3) and aneudiploid *G. tomentella* (E-genome) (Singh et al. 1998b) and *G. canescens* (A-genome). *G. falcata* is morphologically distinct among 26 wild perennial species of the subgenus *Glycine* and two species of subgenus *Soja*. Chromosome pairing result supports the uniqueness of genome (F) of *G. falcata* because it showed minimal chromosome synapsis with A- and B-genomes. Putievsky and Broué (1979) reported a distant relationship between *G. falcata* and *G. clandestina*, and viable hybrids were not obtained with *G. tabacina* ($2n = 40$). Newell and Hymowitz (1983) obtained non-viable hybrids in *G. falcata* × *G. canescens* and *G. falcata* × *G. tomentella* ($2n = 40$) crosses. Cytogenetic studies demonstrate that *G. falcata* does not have a common progenitor present in A, B, C, D, and E-genome species of the subgenus *Glycine* and the origin of this species may be independent, or the genomes are completely distinctly differentiated.

Cytological studies of *G. max*, *G. soja*, and *G. gracilis* revealed nearly identical karyotypes of the three species, confirming their close genetic affinity.

5.8.3 Molecular Studies

Recent studies on biosystematic and phylogenetic relationships of *Glycine* species were carried out applying various molecular technologies, such as RAPD and SSR markers, ribulose biphosphate carboxylase/oxygenase (RuBisCo) *rbcS* subunits, ITS-1 sequences of the rRNA gene, and other genes. Results obtained from these studies consistently demonstrated a significant diversification between species in the subgenus *Soja* and subgenus *Glycine*, although the species relationships of the perennial subgenus showed a more complicated pattern (Cao et al. 1996; Hui et al. 1996; Hui 1997; Wu et al. 2001; Doyle et al. 2003).

Molecular tools verified the cytogenetically based conclusion that *G. max* and *G. soja* are genomically similar (Doyle and Beachy 1985; Doyle 1988; Kollipara et al. 1995, 1997; Zhu et al. 1995). Ribosomal RNA RFLP patterns of *G. max* and *G. soja* appeared to be identical by Doyle (1988) and the sequence divergence for ITS region of rDNA was 0.2% (Kollipara et al. 1997). Broué et al. (1977) were the first to use the isozyme technique to establish genomic relationships among *G. canescens*, *G. clandestina*, and *G. tomentella*. Kollipara et al. (1997) determined phylogenetic relationships among 16 species of the subgenus *Glycine* and two species of the subgenus *Soja* from nucleotide sequence variation in the ITS region of nuclear ribosomal DNA. This study helped to assign genome symbols to five species: H to *G. arenaria*, H_1 to *G. hirticaulis*, H_2 to *G. pindanica*, I to *G. albicans*, and (I_1) to *G. lactovirens*. Cytogenetic relationships among these five species have not been determined because only a few accessions are available and they are difficult to grow in the greenhouse. These genome designations have been verified by histone H3-D gene sequences, and genomes were also assigned to *G. aphyonota* (I_3), *G. peratosa* (A_5), *G. pullenii* (H_3), and *G. stenophita* (B_3) (Brown et al. 2002; Doyle et al. 2002). The ITS region (nrDNA) is a multigene family. However, in the soybean, the nrDNA has been mapped to a single locus on the short arm of chromosome 13 based on the location of the nucleolus organizer region by pachytene chromosome analysis (Singh and Hymowitz 1988) and also by FISH using ITS as a probe (Singh et al. 2001).

5.9 Genetic Resources and Role in Crop Improvement Through Traditional and Advanced Tools

Soybean germplasm collections exist in many countries that contain landraces as well as current cultivars. It is unlikely that many additional landraces will be collected as modern cultivars have replaced most landraces; however, there are likely genotypes of *G. soja* as well as perennial species from the subgenus *Glycine* that remain to be collected. The subgenus *Soja,* which contains the wild annual soybean, *G. soja*,

and the cultivated soybean *G. max*, is considered one genome as hybrids made between accessions of the two species are almost always successful (Table 5.3), viable, and produce fertile F_1 plants. Although all the 26 perennial species of the subgenus *Glycine* could be considered potential sources of useful genes, so far only backcrossed-derived fertile progeny between *G. max* and *G. tomentella* has been reported (Singh et al. 1990, 1993; Singh 2007; Singh unpublished results). Improvements derived from wild species in many crops have enticed crop breeders and researchers to further explore wild genomes in search of beneficial traits. This is easily demonstrated by the literature currently available on work discussing the myriad of beneficial traits of wild relatives and the importance of broadening our crop gene pools. With accumulating information and materials, more efficient approaches to breeding may be developed by merging genomic resources and applied genetics. An example of this new approach is the investigation of genomic diversity in soybeans, followed by identification of genes of agronomic importance. Harlan and de Wet (1971) developed the concept of three gene pools – primary (GP-1), secondary (GP-2), and tertiary (GP-3) – based on the success rate of hybridization among/between species. The clear understanding of taxonomic and evolutionary relationships between a cultigen and its wild relatives is a prerequisite for the exploitation of the primary, secondary, and tertiary gene pools.

5.9.1 Gene Pools

5.9.1.1 Soybean Gene Pools

The primary gene pool (GP-1) for soybean would include cultivars, landraces, and *G. soja* genotypes. GP-1 is defined as biological species that can easily be crossed within the gene pool and produce F_1 hybrids that are vigorous, exhibit normal meiotic chromosome pairing, and possess total seed fertility, such that segregation is normal and gene exchange is basically easy. GP-2 as defined by Harlan and de Wet (1971) consists of species that can be crossed with GP-1 and produce F_1 hybrids that have some fertility. By this definition, there are no currently described *Glycine* species in GP-2. GP-3 is the extreme limit of potential genetic resources classically defined (this does not include transgenes). Harlan and de Wet (1971) suggest that gene transfer is almost impossible or requires rescue techniques that result in sterile (or lethal) hybrids. In *Glycine*, the 26 wild perennial species would be considered GP-3.

Soybean GP-1

Soybean GP-1 consists of biological species that can be crossed to produce vigorous hybrids that exhibit normal meiotic chromosome pairing and possess total seed fertility. Gene segregation is normal and gene exchange is generally easy. Based on this definition, all soybean (*G. max*) germplasm and the wild soybean, *G. soja*, are included in GP-1 (Fig. 5.8) with the qualification that seed sterility can be associated with chromosomal structural changes such as inversions and translocations. Table 5.4 shows the crossability rate in *G. soja* × *G. max*.

Soybean GP-2

GP-2 species can hybridize with GP-1 easily, and F_1 plants exhibit at least some seed fertility (Harlan and de Wet 1971). *G. max* is without GP-2 because no known species has such a relationship with soybean (Fig. 5.8). It is possible that species in the soybean GP-2 do exist in Southeast Asia where the *Glycine* genus may have originated. However, it is merely a speculation, and extensive plant exploration in this part of the world is required to validate this assumption.

Soybean GP-3

GP-3 is the third outer limit of potential genetic resource. Hybrids between GP-1 and GP-3 are lethal, or completely sterile, and gene transfer is not possible or requires radical techniques (Harlan and de Wet 1971). Based on this definition, GP-3 includes the 26 wild perennial species of the subgenus *Glycine*. These species are indigenous to Australia and are geographically isolated from *G. max* and *G. soja* (Fig. 5.8). Table 5.3 shows the *Glycine* species and their 2*n* chromosome numbers, nuclear and plastome genomes,

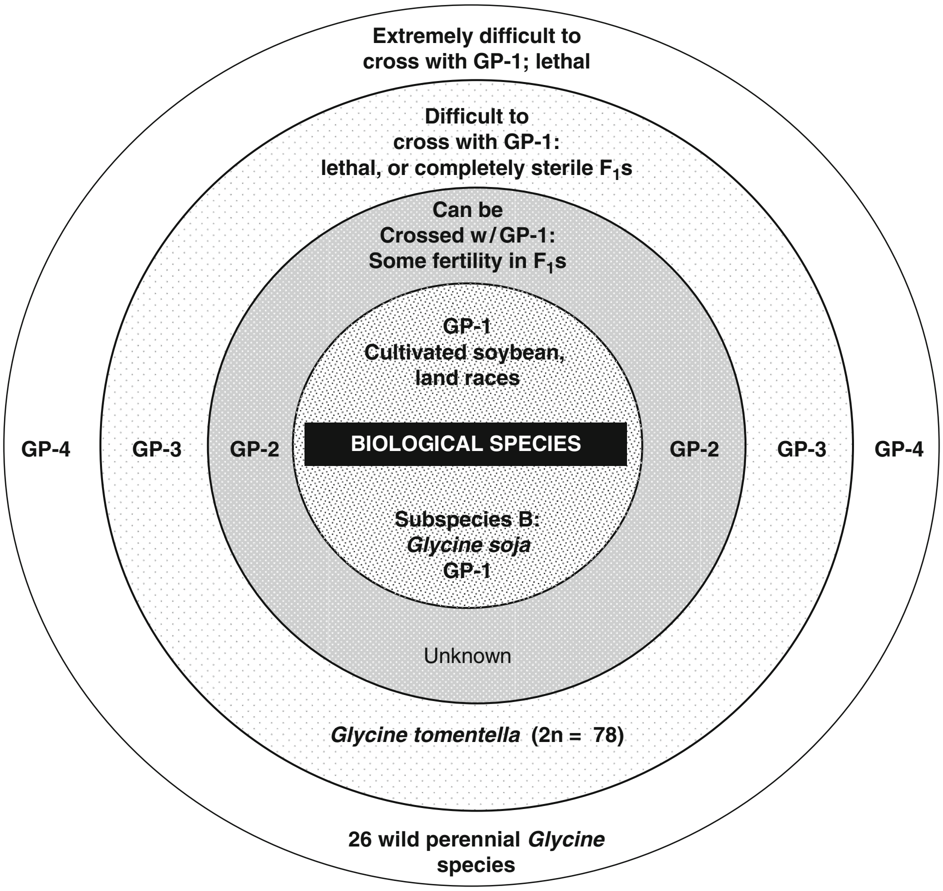


Fig. 5.8 Gene pools of soybean. *GP-1* primary gene pool, *GP-2* secondary gene pool, *GP-3* tertiary gene pool, *GP-4* quaternary gene pool

Table 5.4 Crossability rate in *Glycine soja* ($2n = 40$) × *G. max* ($2n = 40$)

Crosses	No. flowers pollinated	Pod set	Crossability rate (%)	Authors
Bonus × PI 81762	18	2	11.1	Singh and Hymowitz 1988
PI 81762 × Bonus	110	2	1.8	
Essex × PI 81762	46	4	8.7	
PI 81762 × Essex	159	9	6.0	
Glycine soja × *G. max*	139	28	20.14	Singh (unpublished results, 2008)
G. max × *G. soja*	55	8	14.6	

and geographical distributions. Only three species (*G. argyrea*, *G. canescens*, *and G. tomentella*) have been successfully hybridized with soybean (Table 5.5); the F_1 hybrids rescued by embryo culture were sterile, and most researchers could not proceed beyond the amphidiploid stage, with the exception of Singh et al. (1998a) and Singh (unpublished results). This suggests that only three species belong to GP-3.

Soybean GP-4

Harlan and de Wet (1971) did not envision, based on hybridization experiments, the potential existence of a quaternary gene pool (GP-4) because wide hybridization in many crops were not yet attempted, and gene cloning and genetic transformation had not yet been discovered in 1971. GP-4 is the extremely outer

Table 5.5 Progress of wide hybridization in the genus *Glycine*

Number of attempts	Hybrid combinations	Reference
1	TOM ($2n = 38$) × CAN ($2n = 40$); F_1; $2n = 39$ = CT ($2n = 78$) × MAX ($2n = 40$); F_1 sterile	Broué et al. (1982)
2	MAX ($2n = 40$) × TOM ($2n = 78$); F_1; $2n = 59$; sterile	Newell and Hymowitz (1982)
3	MAX ($2n = 40$) × TOM ($2n = 80$); F_1; $2n = 60$; sterile	Newell and Hymowitz (1982)
4	TOM ($2n = 78$) × MAX ($2n = 40$); F_1; $2n = 59$; sterile	Singh and Hymowitz (1985d)
5	MAX ($2n = 40$) × TOM ($2n = 80$); F_1 embryo ($2n = 64$); no F_1 plant	Sakai and Kaizuma (1985)
6	ARG ($2n = 40$) × CAN ($2n = 40$); F_1; $2n = 40$ × MAX ($2n = 40$) = CT ($2n = 80$); sterile	Grant et al. 1986
7	MAX ($2n = 40$) × CLA ($2n = 40$); F_1; $2n = 40$; sterile	Singh et al. 1987a
8	MAX ($2n = 40$) × TOM ($2n = 78$); F_1; $2n = 59$; sterile = CT ($2n = 118$)	Newell et al. (1987)
9	TOM ($2n = 78$) × MAX ($2n = 40$); F_1; $2n = 59$; sterile = CT ($2n = 118$)	Newell et al. (1987)
10	CAN ($2n = 40$) × MAX ($2n = 40$); F_1; $2n = 40$; sterile = CT ($2n = 80$)	Newell et al. (1987)
11	MAX ($2n = 40$) × TOM ($2n = 80$); F_1; $2n$ = Not determined	Chung and Kim (1990)
12	MAX ($2n = 40$) × LAT ($2n = 40$); F_1; $2n$ = Not determined	Chung and Kim (1991)
13	MAX ($2n = 40$) × TOM ($2n = 78$); F_1; $2n = 59$; sterile = CT ($2n = 118$)	Bodanese-Zanettini et al. (1996)
14	MAX ($2n = 40$) × TOM ($2n = 78$); F_1; $2n = 59$; CT = ($2n = 118$) × MAX (BC1–BC6); MAALs	Singh et al. (1990, 1993, 1998a)
15	MAX ($2n = 40$) × TOM ($2n = 78$); F_1; $2n = 59$; CT = ($2n = 118$) × MAX (BC1–BC6); MAALs	Singh (2007, unpublished results)

Abbreviations: TOM *G. tomentella*, CAN *G. canescens*, MAX *G. max*, ARG *G. argyrea*, LAT *G. latifolia*, *CT* Colchicine treatment

limit of potential genetic resources. Pre- and post-hybridization barriers inhibit embryo development, and premature embryo abortion occurs. Rarely can hybrid seedling lethality, hybrid seed inviability, and inviable F_1 plants be circumvented by bridge crosses within the genus *Glycine* (Singh et al. 2007a). Only a few wild perennial *Glycine* species have been hybridized with soybean (Table 5.5). Thus, the majority of species belong to soybean GP-4 as they have not been hybridized with GP-1, or if hybridized, did not produce viable F_1 plants (Singh and Hymowitz 1987). Pods developed when *G. clandestina*, *G. latifolia*, and *G. tabacina* were crossed with soybean but aborted after 19–21 days after pollination. This suggests that the pod abortion in intersubgeneric crosses in the genus *Glycine* is a post-fertilization problem.

Although the wild perennial species carry resistance to several diseases, nematodes, and have tolerance to salt and certain herbicides and lack some biologically active seed components (see Hymowitz 2004 for a detailed listing), the transfer of useful genes into soybean has not been accomplished. Thus, at least for the time being, breeders/geneticists really only have access to the primary gene pool for expanding the germplasm base.

5.9.1.2 Interspecific Hybridization Involving Soybean

Historically, a major limiting factor has been difficulties with interspecific crossability. However, using embryo rescue and other techniques to overcome interspecific crossing barriers, it has been possible to make new hybrid combinations involving different species and to transfer many new traits. Regardless of interspecific crossability, retention of undesirable agronomic traits remains a prominent technical limitation to using wild germplasm. Crosses with wild relatives usually produce lines that have poor agronomic performance, and often, the undesirable traits cannot be eliminated.

Soybean breeders have not fully exploited the wealth of genetic diversity from exotic germplasm including soybean's progenitor *G. soja* (Singh and Hymowitz 1999; Carter et al. 2004; Singh et al. 2007b). *Glycine soja* may be an excellent source of genetic variability (Lee et al. 2008), although it harbors several undesirable genetic traits, for example, vining, lodging susceptibility, lack of complete leaf abscission, seed shattering, and small black coated seeds (Carpenter and Fehr 1986; Carter et al. 2004).

However, *G. soja* has been shown to be more genetically diverse than *G. max* (Li and Nelson 2002; Nichols et al. 2007) and the undesirable traits can be separated from the desirable ones during the course of selection in successive backcross generations and possibly through marker-assisted selection. Attempts to broaden the genetic base of soybeans by utilizing *G. soja* were reported by Hartwig (1973), Ertl and Fehr (1985), Carpenter and Fehr (1986), and Carter et al. (2004). Hartwig (1973) reported highly productive and high-protein lines derived from soybean and *G. soja* hybrids. Ertl and Fehr (1985) concluded that introgression of *G. soja* germplasm into two soybean cultivars was not an effective method for increasing their yield potential. Qian et al. (1996) identified accessions of *G. soja* that are potential sources of additional genes that restrict nodulation of soybean with specific strains of *Bradyrhizobium*. They concluded that introgression of such genes could result in soybean cultivars that exclude some of the indigenous strains and become nodulated with commercial strains that are more efficient at fixing nitrogen.

Several traits have distinct differences between *G. max* and *G. soja* accessions. In general, *G. soja* has much smaller seeds (<3.0 g 100 seeds^{-1}) than *G. max* (generally >9.0 g 100 seeds^{-1}). *G. soja* also has viny and twining stems, severe shattering before plant maturity, and impermeable seed coats, which are all rare in *G. max*. *G. soja* also has much lower oil and oleic acid concentration and higher linolenic acid concentration. *Glycine soja* harbors genes for tolerance to metribuzin [4-Amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one] (Kilen and He 1992), resistance to soybean cyst nematode (Kabelka et al. 2006), and higher seed yield (Concibido et al. 2003), and is a rich source of genetic diversity (Ohara and Shimamoto 2002; Lee et al. 2008, 2010). These invaluable traits could be exploited to broaden the genetic base of soybean. There are many accessions in annual *Glycine* collections that are intermediate between the typical *G. soja* and *G. max* types. Few studies have examined both the genetic and phenotypic relationships among *G. soja*, *G. max*, and semi-wild types (*G. gracilis*) by combining morphological traits and DNA markers.

5.9.1.3 Intersubgeneric Hybridization

Wild perennial *Glycine* species have great potential for soybean improvement. They are a rich source of agronomically useful genes and alleles (Table 5.6). The 26 wild perennial species of the subgenus *Glycine* have not been exploited in soybean breeding programs. These species are extremely diverse morphologically, cytologically, and genomically, grow in very diverse climatic and soil conditions, and have a wide geographical distribution (Singh and Hymowitz 1999). However, extensive and reproducible screening for abiotic and biotic stresses of 26 wild perennial *Glycine* species are lacking. Schoen et al. (1992) studied the resistance of *G. tomentella* to three Australian isolates of soybean leaf rust and found PI 441001 ($2n = 78$) to be resistant to all three races. Researchers hope that the cross between the soybean and this wild relative will produce new types of soybeans that can thrive in dry conditions and are resistant to major diseases, such as rust. It has been demonstrated that resistance to soybean rust in PI 441001 has a chemical basis; a chemical inhibits the growth of fungus spores (Bilgin et al. 2008).

Table 5.6 Source of resistance in the wild perennial *Glycine* species of the subgenus *Glycine*

Traits	Authors
Resistance to soybean rust (*Phakopsora pachyrhizi* Sydow)	Burdon and Marshall (1981), Burdon (1988), Schoen et al. (1992) and Hartman et al. (1992)
Resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe)	Riggs et al. (1998)
Soybean brown spot (*Septoria glycines* Hemmi.)	Lim and Hymowitz (1987)
Powdery mildew (*Microsphaera diffusa* Cke. and Pk.)	Mignucci and Chamberlain (1978)
Tolerance to 2,4-D	Hart et al. (1991)
Resistance to alfalfa mosaic virus	Horlock et al. (1997)
Tolerance to chloride	Pantalone et al. (1997)
Unique nodulation	Pueppke (1988)
Partial resistance to Sclerotinia stem rot and Sudden death syndrome	Hartman et al. (2000)

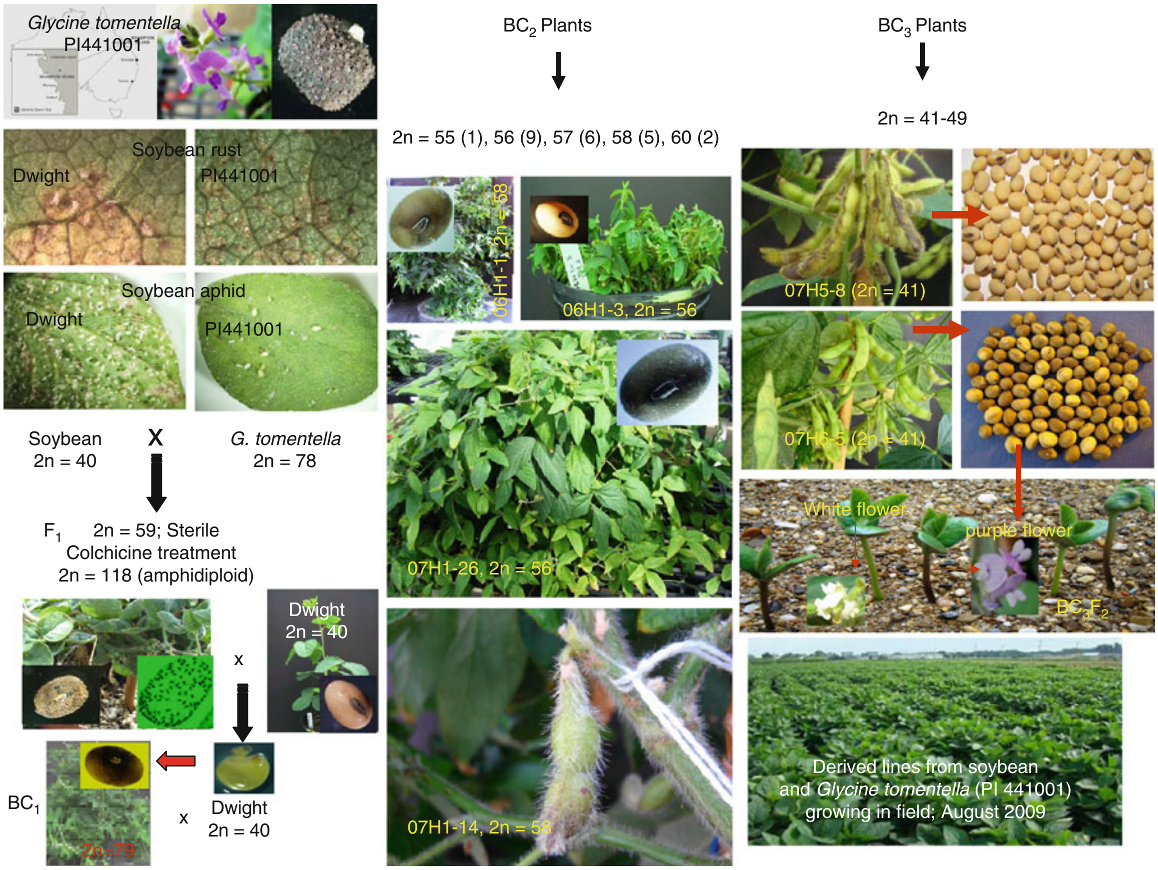


Fig. 5.9 A diagrammatic pictorial scheme to produce fertile soybean lines with $2n = 40$ chromosomes, monosomic alien addition lines (MAALs), and disomic alien addition lines (DAALs) (Singh unpublished results)

Ladizinsky et al. (1979) initiated producing intersubgeneric hybrids between soybean and five wild perennial species of the subgenus *Glycine*. Since 1979, several researchers have attempted to hybridize wild perennial *Glycine* species with the soybean, but only a few (15) sterile intersubgeneric F_1 hybrid combinations have been achieved by using embryo rescue method (Table 5.5). Thus far, only Singh et al. (1990, 1993) have successfully produced backcross-derived fertile progenies from the soybean and a wild perennial, *G. tomentella* ($2n = 78$). Monosomic alien addition lines (MAALs) and modified diploid ($2n = 40$) lines are being isolated and identified (Singh et al. 1998c; Singh 2007; Chung and Singh 2008; Singh unpublished results). A schematic pictorial diagram to produce an intersubgeneric hybrid between soybean and *G. tomentella* is shown in Fig. 5.9. This study has broken the crossability barrier and set the stage for the exploitation of perennial, wild *Glycine* germplasm, a so-called weed from Australia, to broaden the genetic base of the cultivated soybean.

5.9.1.4 Use of *Glycine* Species in Developing Linkage Maps

An accurate and saturated genetic linkage map of soybean is essential for studies on modern soybean genomics, i.e., identification of subtle or new trait loci, including quantitative trait loci (QTLs), map-based cloning, and physical map construction, or even whole-genome sequencing. The first soybean genetic map was constructed with 57 classical markers. Thereafter, molecular maps have been gradually integrated using RFLP, RAPD, SSR, and AFLP markers. In recent years, integrated maps have been reported, each of which was merged from several maps derived from different mapping populations

using JoinMap. Recent advances in DNA sequencing technologies have enabled the production of larger sets of microsatellite and single nucleotide polymorphism (SNP) markers (Cregan et al. 1999; Zhu 2003; Song et al. 2004; Choi et al. 2007; Hisano et al. 2007; Hyten et al. 2007, 2008, 2010a, b; Xia et al. 2007; Shoemaker et al. 2008).

In the initial stage, interspecific mapping populations between *G. max* and *G. soja* were used mainly to increase the number of polymorphic markers. Using a mapping population derived from an interspecific *G. max* × *G. soja* cross, Shoemaker and Olson (1993) developed a molecular genetic linkage map that consisted of 25 linkage groups with about 365 RFLP, 11 RAPD, three classical markers, and four isozyme loci. Subsequently, several intraspecific maps as well as integrated maps have been developed.

Xia et al. (2007) developed an updated genetic map composed of 509 RFLP, 318 SSR, 318 AFLP, 97 AFLP-derived STS (sequence tagged site), 29 bacterial artificial chromosome (BAC)-end or EST derived STS, one RAPD, and five morphological markers, covering a map distance of 3,080 cm in 20 linkage groups (LGs). *G. gracilis*, which originated in Northeast China, is morphologically intermediate between the cultivated *G. max* and the wild form, *G. soja*, and has been used to construct a soybean genetic map. Crosses between the cultivar (Misuzudaizu) and the intermediate form *G. gracilis* (Moshidou Gong 503) provides good genetic resources for linkage map construction and for the isolation of agronomically and biologically important genes. A framework of genetic linkage map had been previously constructed mainly with RFLP and SSR markers using a single F_2 population of this combination (Yamanaka et al. 2000, 2001).

The haploid chromosome number of the soybean plant is 20 (Veatch 1934), which is almost two times that observed in major diploid crops such as rice (12), maize (10), barley (7), and tomato (12) as well as in model plants including *Arabidopsis thaliana* (5), *Lotus japonicus* (6), and *M. truncatula* (8). The soybean mitotic metaphase chromosomes evidence small size variations ranging from 1.42 to 2.84 μm and are symmetrical without karyotypically visible landmarks (Sen and Vidyabhusan 1960). However, the pachytene chromosome analysis of an F_1 hybrid between soybean and *Glycine soja* Sieb. and Zucc. evidenced heterochromatin distribution on either side of the centromeres, small structural differences, and a satellite chromosome, thus allowing for the construction of chromosome maps on the basis of chromosome length and euchromatin and heterochromatin distribution, which were numbered in descending order of 1–20 (Singh and Hymowitz 1988). The chromosome harboring the satellite was designated as chromosome 13 (Singh and Hymowitz 1988). Fluorescent in situ hybridization (FISH) using the rDNA as a probe verified that the satellite region of chromosome 13 is the nucleolar organizer region (NOR) (Griffor et al. 1991). FISH resulted in the detection of a pair of NORs on the short arm of chromosome 13 in the soybean and its progenitor, *G. soja*, as well as three strong fluorescent signals in the soybean that are trisomic for chromosome 13. Subsequently, MLG F was assigned to chromosome 13 with a set of primary trisomics and SSR markers (Cregan et al. 2001). However, the accurate genetic location of the soybean NOR on MLG F remains to be determined. Recently, Yang and Jeong (2008) developed a genetic linkage map of the nucleolus organizer region in the soybean.

5.10 Genomic Resources

Perhaps the most promising in their potential to increase the use of wild relative genes are the advancements in the field of genomics. While introgression was not easily detectable with the genetic tools of a few decades ago, recent use of DNA markers and sequencing has helped in isolating beneficial genes and in selecting for traits, which are difficult to detect based on phenotype. Soybean is now the first legume species with a complete genome sequence. It is, therefore, a key reference for the more than 20,000 legume species, and for the remarkable evolutionary innovation of nitrogen-fixing symbiosis. The genome sequence is an essential framework for vast new experimental information such as tissue-specific expression and whole-genome association data (Schmutz et al. 2010a, b).

Comparative genomics may represent another promising means of utilizing genomic resources for applied genetics. BAC library resources have been developed for the genus *Glycine*. BAC libraries have been constructed for eight genome types (species) within the genus *Glycine* (Schmutz et al. 2010b). These species represent nearly 5 million years of

evolution and a diversity of genotypes and ecological adaptations within the genus. BAC-end sequences (BESs) are being produced so that the BACs can be aligned to the reference soybean genome to provide a resource for rapid transition between *Glycine* genomes to clone genes, understand sequence variation, explore domestication, and introgress new genetic diversity into the soybean gene pool. Libraries have been constructed for *G. soja*, *G. syndetika*, *G. canescens*, *G. stenophita*, *G. cyrtoloba*, *G. tomentella*, *G. falcata*, and the polyploid, *G. dolichocarpa*. All libraries are publicly available though the Arizona Genome Institute and are part of an NSF Plant Genome project to leverage diversity within the genus *Glycine*.

Innes et al. (2008) sequenced an approximately 1 Mbp region in soybean centered on the *Rpg1-b* disease resistance gene and compared this region with a region duplicated 10–14 Mya. These two regions were also compared with homologous regions in several related legume species (a second soybean genotype *G. tomentella* D3, *Phaseolus vulgaris*, and *M. truncatula*). In this study, a set of 36 BACs were sequenced from both diploid and tetraploid *G. tomentella* (*http://sites.bio.indiana.edu/~nsflegume/progress.php*). Comparison of ~1 Mb region of soybean with the orthologous regions of *G. tomentella* D3 ($2n = 40$) addressed several fundamental questions relating to Nucleotide binding-Leicine rich repeat (NB-LRRs) genes, polyploidy, and genome evolution. Analysis revealed a high level of conservation of low-copy genes but major differences in the NB-LRR content and retroelement content as well as differences in copy number of a family of protein kinases. Low-copy gene order of *G. tomentella* D3 is nearly identical to that of soybean. Analysis of 15 conserved gene pairs gave a mean Ks value of 0.064 ± 0.034, consistent with a divergence time of 5–7 Mya for these two species. Interestingly, there are examples of low-copy gene loss unique to *G. tomentella* D3 not observed in soybean, indicating that homoeolog-specific gene loss has continued subsequent to the divergence of these two species, albeit at a slow rate.

Retrotransposons and their remnants often constitute more than 50% of higher plant genomes. Although extensively studied in monocot crops such as maize and rice, the impact of retrotransposons on dicot crop genomes is not well documented. Wawrzynski et al. (2008) identified several retrotransposon families in the genomes of *G. tomentella* and grouped the 23 intact elements into 16 families. Nine of these 16 families contain elements that had inserted within the last million years, and two elements contained identical long terminal repeats (LTRs). In addition to this, several apparently replicating non-autonomous retrotransposon families were identified. The results indicated that autonomous and non-autonomous retrotransposons appear to be both abundant and active in the *soybean* and *G. tomentella* genome.

Gill et al. (2009) characterized and analyzed two subfamilies of high-copy centromeric satellite repeats, CentGm-1 and CentGm-2, using a combination of computational and molecular cytogenetic approaches. These two subfamilies of satellite repeats mark distinct subsets of soybean centromeres and, in at least one case, a pair of homoeologs, suggesting their origin from an allopolyploid event. These satellite repeats are also present in *G. soja*, the wild progenitor of soybean, but could not be detected in any other relatives of soybean examined in this study, suggesting the rapid divergence and species-specific concerted evolution of the centromeric satellite DNA within the *Glycine* genus.

The development of a universal soybean cytogenetic map that associates classical genetic linkage groups, molecular linkage groups, and a sequence-based physical map with the karyotype has been impeded due to the soybean chromosomes themselves, which are tiny and morphologically homogeneous. To overcome this obstacle, Findley et al. (2010) screened soybean repetitive DNA to develop a cocktail of fluorescent in situ hybridization probes that could differentially label mitotic chromosomes in root tip preparations. Karyotyping tools were applied to wild soybean, *G. soja* Sieb. and Zucc., which represents a large gene pool of potentially agronomically valuable traits. These studies led to the identification and characterization of a reciprocal chromosome translocation between chromosomes 11 and 13 in two accessions of wild soybean.

ESTs, which are generated by large-scale single-pass sequencing of randomly picked cDNA clones, have proven to be an efficient and rapid means to identify novel genes. Ji et al. (2006) sequenced 2,003 ESTs generated from salinity-treated *G. soja* cDNA library, putatively representing 1,071 unigenes. Comparison of *G. soja* ESTs with those of *G. max* revealed the potential to investigate the wild soybean's

expression profile using the soybean's gene chip. Through analysis of the ESTs with putative functional annotations, a large number of putative stress-regulated genes were identified. In the genus *Glycine*, many large-scale EST sequencing projects are in progress, and comparative genomic studies are being initiated.

A major milestone in soybean research was the recent sequencing of its genome. The sequence predicts 69,145 putative soybean genes, with 46,430 predicted with high confidence. Libault et al. (2010) utilized the Illumina Solexa platform to sequence cDNA derived from 14 conditions (tissues). The result is a searchable soybean gene expression atlas accessible through a browser (*http://digbio.missouri.edu/soybean_atlas*). The data provide experimental support for the transcription of 55,616 annotated genes.

MicroRNAs (miRNAs) play important roles in post-transcriptional gene silencing by directing target mRNA cleavage or translational inhibition. Currently, hundreds of miRNAs have been identified in different plant species. Chen et al. (2009) constructed a small-RNA library consisting of 2,880 sequences and analyzed the secondary structure of these small RNAs. As a result, 15 conserved miRNA candidates belonging to eight different families and nine novel miRNA candidates comprising eight families were identified in wild soybean seedlings. These results provided useful information for miRNA research in wild soybean and plants.

5.11 Scope for Domestication and Commercialization

Soybean is widely used as food, feed, and for industrial purpose (Fig. 5.10). Among the wild *Glycine* species, *G. tomentella* is grown as a commercial crop in Taiwan, with their roots being used as the source of the herbal drug, I-Tiao-Gung, which is used as an important component of many herbal health products. It has been used to help treat arthritis and rheumatism for a long time. Recently, the herb has also been exploited in Taiwan as a functional tea, as steeped wine, and as a related product for commercial purposes, due to its health benefits. It has been reported that the roots of *G. tomentella* Hayata have several biological activities, such as antioxidant hypolipidaemic, analgesic, anti-inflammatory and immunomodulatory (Chen et al. 2005; Pan et al. 2005; Chuang et al. 2008). In addition to this, *G. tabacina* roots have also

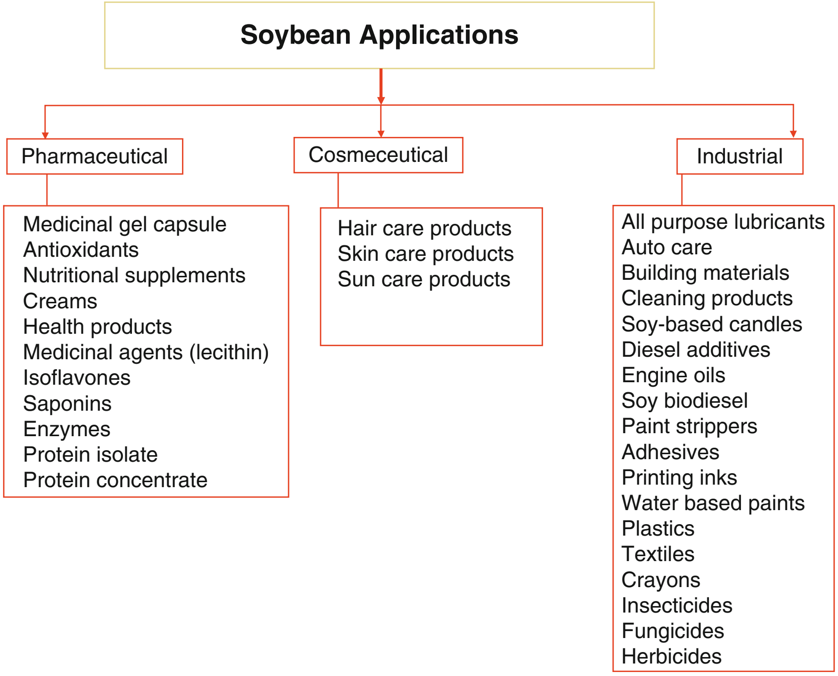


Fig. 5.10 Application of soybean for industrial purpose

been used for many years as a herbal drug although little work has been done to identify its constituent compounds.

Isoflavones, naturally occurring plant compounds found almost exclusively in soybeans and other leguminous plants, have been intensively studied in recent years with regard to their substantial health benefits. Isoflavones belong to a group of plant compounds with potential anticarcinogenic properties. Acting as antioxidants and tyrosine protein kinase inhibitors, they may lower the risk of cardiovascular disease and breast cancers. Contents of certain isoflavones in *G. dolichocarpa*, *G. tabacina*, and *G. tomentella* collected in Taiwan were reported by Lin et al. (2005). While little information is available about the therapeutic uses of the other wild *Glycine* species, they may be viewed as a source of value-added traits to enhance the nutritional and medicinal benefits of commercial varieties for human and animal populations.

5.12 Some Dark Sides and Potential Solutions

Modern soybean breeding procedures with intensive selection pressure have led to a dangerously narrow genetic base of the currently improved soybean varieties. The extensive cultivation of a few high-yielding soybean varieties on an exceptionally massive scale has caused tremendous genetic erosion of the soybean gene pool. Due to the rapid economic development, urbanization, industrialization, and expansion of transportation systems, many habitats of wild soybean have significantly deteriorated, leading to the extinction or decrease of many wild soybean populations.

The diversity of wild relatives of crop species with high international significance is increasingly threatened by land conversion, improper agricultural practices, intensification of land use, spread of invasive species, pollution, and the impact of genetically modified crops (Lu 2004). The underlying causes of these threats include short-term economic development measures at the local level, institutional constraints to implementation of conservation regulations, promotion of new cultivars and new techniques by the agricultural extension system, and obscurity of population status of wild relatives. If unaddressed, these issues will result in loss of genetic resources for the world. Conservation of biodiversity in the soybean gene pool, particularly in its origin and diversity center, is facing severe challenges.

Gene flow via pollination is a natural process whereby genes, aided by wind or insects, are exchanged between plants. This process can occur between sexually compatible plants and wild relatives if the appropriate conditions are met. In the case of crop plants, this process can occur regardless of whether the crop plant was developed through conventional plant breeding or biotechnology. In the United States and Canada, crops, such as maize and soybeans, do not have wild relatives nearby, so gene flow is limited to neighboring cultivated plants within the same field or to nearby fields. The likelihood of gene flow diminishes when the plants are located farther apart, even if they are in the same field or region. For some crops such as soybeans, pollination characteristics limit gene exchange even between neighboring plants in the same field. Gene flow to wild relatives is not an important issue for soybeans growing in the US, Canada, or Europe, but becomes potentially more important for soy grown in certain parts of China and Siberia, Taiwan, Korea, Japan, and Australia. Unlike maize, the relatives of soybean self-pollinate, or fertilize themselves, before the flower even opens. This means that the chance of other pollen fertilizing a plant is low. In addition, gene flow would depend on a number of other factors, though the factors that further determine the extent of gene flow are less relevant for soybean than maize. Transgenic soybean is grown on a larger area globally than any other transgenic crop, but it is not currently grown in Asia where the wild progenitor of soybean, *G. soja*, grows. The release of such varieties to the environment, particularly to the origin and diversity centers of soybean and wild soybean species, might cause a significant change in the general diversity patterns in traditional soybean landraces and wild soybean populations (Lu 2004). The spread of transgenes into the genome of wild soybean is a concern when transgenic and wild soybeans are planted sympatrically. Kuroda et al. (2010) investigated the origin and fate of morphological intermediates between wild and cultivated soybeans in their natural habitats in Japan. The chloroplast DNA haplotypes revealed that all intermediate soybean plants originated from gene flow from cultivated to wild soybeans at all sites. Based on monitoring at both the phenotypic and molecular levels, hybrids quickly

disappeared from natural habitats, and secondary gene flow from these plants to wild soybean was not detected. Thus, while gene flow from transgenic soybean into wild soybean can occur, gene introgression appears to be rare in natural habitats in Japan.

5.13 Recommendation for Future Actions

Despite the challenges of working with a relatively large and complex genome, the construction of genetic linkage maps in soybean has come a long way over the last 20 years. Currently, the number of linkage groups for soybean is 20, corresponding to its haploid chromosome number, and the total map length ranges from approximately 2,500 cm to 3,000 cm. These numbers strongly suggest that the consensus linkage map of soybean has already been saturated. Sequencing of the entire soybean genome is complete, which provides a substantial amount of genome sequence as well as physical maps of the entire genome. By taking advantage of these new data, integration of the genetic linkage map and the physical map has been attempted. The genetic map of the soybean is one of the most densely populated maps among plants, with >4,000 published markers. However, its cytogenetic studies have lagged behind those of rice, maize, barley, and tomato. Thus, the relationships between soybean molecular linkage groups (MLG) and chromosomes remain incompletely understood (Cregan et al. 2001; Zou et al. 2003; Chung and Singh 2008). Twenty possible primary trisomics (Singh and Hymowitz 1991; Xu et al. 2000b), two monosomic (Xu et al. 2000a) and tetrasomics (Chung and Singh 2008) in soybean have been developed and results have been published. A major effort is recommended for developing a universal cytogenetic map for soybean.

Wild *Glycine* species are a rich source of agronomically useful genes and alleles and have great potential for soybean improvement. With the availability of effective genetic recombination and engineering technology, genetically modified (GM) soybean varieties would be developed with considerable speed. There are still many scientific questions relating to the ecologic consequences of transgene escape to wild soybean populations, which need to be thoroughly studied (Lu 2004). It is, therefore, recommended that people should be more cautious regarding the issues of transgene escape and its environmental consequences before extensively releasing GM soybean into the environment of its origin and diversity centers.

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Chapter 6
Lathyrus

Allison M. Gurung and Edwin C. K. Pang

6.1 Introduction

Lathyrus is a member of the Vicieae tribe (family Fabaceae), the other members of which are *Pisum*, *Lens*, and *Vicia*. There are approximately 160 species in the genus *Lathyrus* (Allkin et al. 1986); thus, there is potentially an enormous wealth of genetic diversity in the genus. Of the *Lathyrus* species, the most economically important and widely cultivated crop for human consumption is *L. sativus* L. Other *Lathyrus* species, which are grown for forage and/or grain include *L. cicera*, *L. ochrus*, *L. clymenum*, *L. tingitanus*, *L. latifolius*, and *L. sylvestris* (IPGRI 2000). In addition, *L. odoratus* (sweet pea) is widely cultivated as an ornamental plant.

The center of origin and main center of diversity of *Lathyrus* species is the Eastern Mediterranean, with smaller centers of diversity in North and South America (Kupicha 1983). Archeological remains of *L. sativus* seeds have been found dating back to 8000 BC and 6000 BC from the Balkans (Kislev 1989) and Jarmo (Iraq) (Helbaek 1965), respectively. *L. sativus* (grasspea) is now widely cultivated in South Asia and Ethiopia, with smaller areas of cultivation in other countries, including Spain, China, and Chile (IPGRI 2000). *L. sativus* is suited to a range of environments from temperate to subtropical regions. It is very tolerant of drought conditions and is also not affected by excessive rainfall (Campbell et al. 1994). However, despite the widespread distribution of *L. sativus* and the length of time that it has been cultivated, it has not progressed as a pulse crop to the same extent as other pulses including field pea, lentil, and chickpea. Smartt (1984) hypothesized that the lack of progress of *L. sativus* as a pulse crop might have been due to its other and perhaps more important use as a forage crop.

L. sativus is an herbaceous annual (Smartt 1984); however, the genus *Lathyrus* also includes perennial species. Morphology of *L. sativus* genotypes is described in detail by Campbell (1997). International descriptors for *Lathyrus* species are based on diversity observed for the three most widely cultivated *Lathyrus* species: *L. sativus*, *L. cicera*, and *L. ochrus* (IPGRI 2000). The five categories of descriptors are: passport, management, environment and site, characterization, and evaluation. These descriptors are designed as a tool for a standardized characterization system and will assist with conservation of genetic resources.

Taxonomic characters that have traditionally been used to classify *Lathyrus* species include the anatomy of stipules, stems and phyllodes, number of leaflets, leaf venation, epidermal cell shape, flower structure, flower density, and legume shape (Kupicha 1983). Classification of *Lathyrus* based on these morphological traits separated the species into 13 sections (Orobus, Lathyrostylis, Lathyrus, Orobon, Pratensis, Aphaca, Clymenum, Orobastrum, Viciopsis, Linearicarpus, Nissolia, Neurolobus, and Notolathyrus) (Kupicha 1983).

All known annual *Lathyrus* species and most perennial species in *Lathyrus* are diploid with $2n = 14$ chromosomes. There are a few polyploids among the perennials, including *L. palustris*, a hexaploid with $4x = 42$, and *L. venosus*, a tetraploid with 28 chromosomes (Narayan and Durrant 1983; Gunes and Ali 2008). Large increases in chromosome size have occurred during the evolution of diploid *Lathyrus* species; Narayan and Durrant (1983) measured a fourfold difference in DNA between 25 *Lathyrus* species.

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However, despite differences in chromosome size, their shape and karyotype arrangement between complements are similar among *Lathryus* species (Narayan and Durrant 1983). Although trisomic and tetrasomic types of *L. sativus* have been recovered (Dibyendu 2008), there remains limited information on their use. The tetrasomics varied in morphological characters and had reduced pollen sterility and yield (Dibyendu 2008). Natural and induced autoployploids have also been reported for *L. sativus*, *L. odoratus*, *L. pratensis*, and *L. venosus* (Khawaja et al. 1997).

There have been several comprehensive reviews of the conservation of *Lathyrus* (Campbell 1997; Sarker et al. 2000; Heywood et al. 2007) and the status of *Lathyrus* improvement using classical breeding and molecular techniques (Vaz Patto et al. 2006a; Skiba et al. 2007). Therefore, in this chapter, we will discuss the more recent developments as a supplement to these previous publications.

6.2 Conservation Initiatives

Coordinated evaluation and conservation of *Lathyrus* genetic resources has attracted more attention in the last 10–15 years. In 1995, a regional workshop on *Lathyrus* genetic resources in Asia was organized by the International Plant Genetic Resources Institute (IPGRI) and Indira Gandhi Agriculture University in India. The workshop proposed a working group to follow-up on the suggested activities of a regional network based on *Lathyrus* genetic resources conservation and use (Mathur et al. 1998). A follow-up regional working group meeting was held in 1997 in New Delhi, India, with the proceedings published under the title "*Lathyrus* Genetic Resources Network" (Mathur et al. 1998). The main focus of the network was *L. sativus*, although *L. cicera* and *L. ochrus* were a secondary focus. Recently, the development of a grasspea conservation strategy has been supported by the Global Crop Diversity Trust, which manages the Svalbard Global Seed Vault. The grasspea strategy focuses on the ex situ conservation of *L. sativus*, *L. cicera*, and *L. ochrus* (Hawtin 2007). The conservation strategy document prepared for *Lathyrus* details the current status of national collections and identifies gaps in collections of these three species from areas of diversity. The strategy recommends that documentation on collections be upgraded and that more work be carried out on characterizing and evaluating collections for key traits and making this data widely available.

To date ex situ *Lathyrus* conservation has primarily focused on collecting germplasm of cultivated species of *Lathyrus*, primarily *L. sativus*, and there has been very little attention paid to the conservation of other *Lathyrus* wild species. Countries including France and Bangladesh hold a large number of *Lathyrus* accessions, but the collections are limited to a few species, predominantly *L. sativus* (Sarker et al. 2000; Table 6.1). The largest ex situ collections of *Lathyrus* germplasm, based on the number of species, are held by ICARDA (44 species), USA (41 species), Australia (38 species), India (34 species), and Turkey (31 species) (Sabanci 1996; Robertson and El-Moneim 1998; Sarker et al. 2000, 2001; Mathur et al. 2005). Although exploitation of genetic diversity within *L. sativus* still holds much potential, it is crucial to also conserve the genetic diversity in the wild species for future exploitation, particularly as a source of potentially novel traits.

Centers of primary and secondary diversity of a species are priority collection regions; however, examination of national collections from centers of *Lathyrus* diversity show that some of the collections do not contain a large proportion of indigenous accessions (Table 6.1). In addition, sharing of *Lathyrus* accessions between nations can give the appearance that collections contain more resources than in reality; for example, 87% of the ICARDA collection is duplicated in other collections. Important gaps in ex situ genetic diversity of *L. sativus* were identified in Egypt, Iran, and regions of Russia, Iraq, Bangladesh, India, Ethiopia, Afghanistan, and Spain (Hawtin 2007). In addition to focusing on expanding collections, duplication of collections needs attention for safety purposes. The grasspea conservation strategy found less than 10% duplication of accessions for some important collections (Hawtin 2007).

In situ conservation of *Lathyrus* is not formally practiced; however, naturally occurring areas of *Lathyrus* still exist, although their survival is threatened by genetic erosion due to intensification of agriculture and threatened habitats. Five genetic reserves for *Lathyrus* diversity have been proposed in Syria and Turkey (Maxted 1995; Heywood et al. 2007). These sites were found to have many rare and diverse

Table 6.1 *Lathyrus* germplasm in national collections

Country	*L. sativus*	*L. amphicarpos*	*L. cicera*	*L. gorgoni*	*L. latifolius*	Total *Lathyrus* accessions	Indigenous accessions
Algeria[a]	234	0	45	1	0	437	26
Australia[a]	583	0	141	6	1	1,001	0
Bangladesh	2,432	0	0	0	0	2,432	2,422
Bulgaria[b]	213	0	44	0	0	369	ns
Cyprus[a]	19	0	0	0	0	31	31
Ethiopia[a]	115	0	2	0	0	163	5
France[a]	2,345	0	776	0	308	4,387	1,467
Germany[a]	205	2	61	2	4	445	4
Hungary[a]	228	0	55	0	0	307	101
India[a]	2,561	0	1	0	0	2,580	2,453
Jordan[a]	1	0	1	1	0	36	35
Nepal[a]	149	0	0	0	0	149	144
Pakistan[a]	11	0	0	0	0	130	55
Russia[c]	688	0	57	2	0	904	131
Spain[b]	178	0	220	0	1	442	275
Turkey[d]	17	0	90	27	0	>600	>600
USA[a]	242	0	33	1	11	529	48
ICARDA[e] (1998)	1,627	2	183	60	1	3,038	519[f]
ICARDA[g] (2007)	1,660	ns	208	ns	ns	3,239	ns

[a]Mathur et al. (2005) (Indian collection: Department of Plant Breeding and Genetics Indira Gandhi Agricultural University, Raipur)
[b]EURISCO Catalog (*http://eurisco.ecpgr.org*, date of data consultation 25 May 2009)
[c]Database of Vavilov Institute (*http://www.vir.nw.ru/data/dbf.htm* accessed 25 May 2009)
[d]Sabanci (1996)
[e]Robertson and El-Moneim (1998)
[f]Indigenous accessions from Syria in the ICARDA collection
[g]Hawtin (2007)

Lathyrus species (Maxted 1995). The importance of these types of sites is demonstrated by the recent finding of a new species of *Lathyrus* in Turkey, *L. egirdiricus* (Genc and Sahin 2008).

6.3 Elucidation of Origin and Evolution of Allied Crop Plants

L. sativus is classified in Section *Lathyrus* with 33 other species (including *L. amphicarpos*, *L. cicera*, *L. gorgoni*, *L. odoratus*, and *L. latifolius*) according to morphotaxonomic characters (Kupicha 1983). A biochemical study of free amino acids in the seeds of 50 *Lathyrus* species by Bell (1962) found that the species could be subdivided into five related groups (*L. clymenum*, *L. ochrus*, *L. sativus*, and *L. cicera* in Group I, *L. gorgoni* in Group II, and *L. odoratus* in Group V), which only partially corresponded with the morphological study (Kupicha 1983). Studies of the chloroplast DNA of 42 *Lathyrus* species (Asmussen and Liston 1998) and amplified fragment length polymorphism (AFLP) analysis of 18 species (Badr et al. 2002) suggest that reclassification of some species to different sections may be required. In comparison, random amplified polymorphic DNA (RAPD) analysis of eight species (from three sections) supported the classification system of Kupicha (Croft et al. 1999). Examination of 53 species using internal transcribed spacer (ITS) and 5.8S coding region of nuclear ribosomal DNA also generally supported the morphological classification of *Lathyrus* species, particularly in Section *Lathyrus* (Kenicer et al. 2005).

The wild *Lathyrus* species that comprise the most accessible gene pool of *L. sativus* are the species with which *L. sativus* produces hybrids with some degree of fertility. Within Section *Lathyrus*, morphological variation (Jackson and Yunus 1984) and interspecific hybridization (Yunus and Jackson 1991) have been examined to identify the species most closely related to *L. sativus*. Morphological characterization of 14 species from Section *Lathyrus* indicated that *L. cicera*

and *L. gorgoni* were most closely related to *L. sativus* (Jackson and Yunus 1984). Interspecific hybridization between *L. sativus* and 15 wild species in Section *Lathyrus* supported the close relationship between *L. sativus* and *L. cicera*, with crossing between these species resulting in viable F_1 hybrids with low fertility (Yunus and Jackson 1991). Viable hybrids were also obtained between *L. amphicarpos* and *L. sativus* (Yunus and Jackson 1991). *L. gorgoni* × *L. sativus* produced an F_1, but the seedling was nonviable. Embryo abortion was an important barrier to interspecific hybridization between many of the Section *Lathyrus* species (Yunus and Jackson 1991); thus, ovule culture and embryo rescue techniques may be successful in increasing the success and efficiency of interspecific hybridizations between *L. sativus* and other Section *Lathyrus* species. Embryo rescue techniques for *Lathyrus* have been used successfully to produce hybrids between *L. odoratus* and *L. belinensis* in an attempt to transfer unique flower color traits to *L. odoratus* (Hammett et al. 1994), and Kearney (1993) also reported in a PhD thesis that ovule culture and embryo rescue techniques were successful in increasing the success and efficiency of interspecific hybridizations between *L. sativus* and other Section *Lathyrus* species.

Thus, in terms of conservation of *Lathyrus* wild relatives as a source of genetic variation for *L. sativus* that may be accessible using traditional interspecific hybridization techniques, *L. cicera* and *L. amphicarpos* are the most important species as they make up the secondary gene pool, followed by other Section *Lathyrus* species that make up the tertiary gene pool. A summary of national collections of a few Section *Lathyrus* species in Table 6.1 shows that relatively large collections of *L. cicera* exist in a number of countries due to its agricultural use. Thus, *L. cicera* represents a valuable source of potentially accessible variation for *L. sativus*. In comparison, only a couple of accessions of *L. amphicarpos* exist and they were collected more than 30 years ago (the two accessions in the German collection were acquired in 1962 and 1977 from Portugal; Table 6.1). In light of successful hybridizations between *L. amphicarpos* and *L. sativus*, it would be worthwhile revisiting the taxonomic status of *L. amphicarpos* and confirming it is a separate species.

The wild *Lathyrus* species represent a wealth of genetic variation that is potentially available to *Lathyrus* crop species, but the wild species may also be a source of novel genes that can be accessed using modern genetic engineering technology. However, few accessions exist of *Lathyrus* species that are not agriculturally important. For example, less than 100 accessions of *L. gorgoni* are maintained, and it is likely that a significant proportion of these are duplicated (Table 6.1). Thus, conservation initiatives for the wild *Lathyrus* species need to be expedited before potentially valuable sources of genetic variability are permanently lost.

6.4 Crop Improvement Through Traditional and Advanced Tools

Traditional and molecular breeding of *Lathyrus* were reviewed comprehensively by Vaz Patto et al. (2006a) and Skiba et al. (2007); therefore, the reader is directed to these texts. In this section, we propose to discuss more recent developments as a supplement to these previous publications. Briefly, the major breeding objective for *L. sativus* improvement is reducing concentrations of the neurotoxin β-*N*-oxalyl-L-α,β-diaminopropanoic acid (ODAP) in the seed. ODAP is a nonprotein amino acid associated with a neurodegenerative disease called neurolathyrism. The disease causes a degeneration of upper motor neurons, which is manifested as irreversible spastic paraparesis of the lower limbs (Spencer et al. 1986). *L. sativus* germplasm exhibits a large natural range of variation in ODAP concentrations in seed, ranging from ca. 0.22 to 7.20 g/kg (Campbell 1997), and somaclonal variation for the trait has also been observed (Santha et al. 1998; Chakrbarti et al. 1999).

Other major breeding objectives for *L. sativus* improvement are: increasing grain yield through the incorporation of yield components such as double pods per node and increased seeds per pod and increasing biomass yield (for forage varieties) (Campbell 1997; Abd El Moneim et al. 2001). Vaz Patto et al. (2006a) showed that previous breeding efforts have concentrated on the production of low β-ODAP lines, and it was only after the release of such lines that other traits, e.g., yield and abiotic/biotic stress resistance have been considered. However, progress in these areas has been slow, as no new report has

emerged recently of further advancements in breeding of such traits.

Other *Lathyrus* species have not fared as well as *L. sativus* in terms of breeding research, although there is the potential for using the significant variation in the *Lathyrus* secondary and tertiary gene pools for the improvement of *L. sativus*. Resistance to powdery mildew (*Erisiphe pisi*) in *Lathyrus* has been investigated in hybrid sweet peas (*L. odoratus* × *L. belinensis*) by Poulter et al. (2003) and recently for *L. sativus* (Vaz Patto et al. 2006b). Full and partial resistance to this disease has been observed, indicating that qualitative (major gene) and quantitative (minor gene) inheritance may be involved. Recently, a collection of 140 accessions of *L. cicera* from Iberia were characterized for rust (*Uromyces pisi* and *U. viciae-fabae*) resistance (Vaz Patto et al. 2009). Although most accessions displayed only partial resistance to *U. pisi*, they were, in general, found to be highly resistant to *U. viciae-fabae*, as evidenced by clear hypersensitivity responses. Vaz Patto et al. (2006b) identified other areas of breeding research for *Lathyrus*, including downy mildew (*Peronospora lathyri-palustris*) resistance in *L. aphaca* and *L. sativus* (Campbell 1997), Ascochyta blight (*Mycosphaerella pinodes*) resistance in *L. sativus* (Gurung et al. 2002; Skiba et al. 2004), and broomrape (*Orobanche crenata*) resistance in *Lathyrus* spp. A search of the available literature revealed no recent progress in these areas.

Molecular breeding in *Lathyrus* is at a nascent stage. Although several marker systems have been developed for *Lathyrus* (Skiba et al. 2007), none has been employed for regular marker-assisted breeding. Most of the DNA-based marker systems reviewed by Skiba et al. (2007) were employed for genetic diversity and phylogenetic determinations; few studies were directed toward the development of markers for traits of economic importance. As an example, one of these latter studies involved the development of a molecular marker linked to the tendril trait for *L. odoratus* (Hanada and Hirai 2003). The region in the *L. odoratus* genome that was tagged by a RAPD marker linked to the tendril trait was cloned and sequenced, and a pair of primers was designed to specifically amplify this region, thereby developing a sequence-characterized amplified region (SCAR) marker. Further, Skiba et al. (2004) developed 13 sequence tagged site (STS/CAPS) markers from expressed sequence tag (EST) sequences obtained from a *L. sativus* cDNA library. These markers, representing a variety of genes, were subsequently mapped onto a linkage map of *L. sativus*. A quantitative trait loci (QTL) for Ascochyta blight resistance mapped closely to a sequence tagged site (STS) for a Cf-9 homolog (*Cladosporium fulvum R*-gene homolog), suggesting the possible involvement of this gene in the defense response. However, no subsequent proof-of-function research was performed to validate these results; so currently, the *Cf-9* homolog may only be regarded as a molecular marker for Ascochyta blight resistance but not as an effector of resistance.

Thus far, only two linkage maps are available for *Lathyrus*, both of them developed for *L. sativus* independently by Chowdhury and Slinkard (1999) and Skiba et al. (2004). The first of these maps was constructed with mainly dominant RAPD markers (Chowdhury and Slinkard 1999). This map consisted of 71 RAPD, three isozymes and a morphological marker organized into 14 linkage groups spanning 898 cm, with an average marker distance of 17.2 cm. However, it is clear that this genetic map does not align properly with the physical map of *Lathyrus* as its haploid chromosome number is $n = 7$. This may have been due to the high level of segregation distortion of the RAPD markers (12%), as the use of such markers in map construction may lead to false linkages and unreliable estimates of map distances. Additionally, the segregation distortion of these markers may have been symptomatic of the overall level of heterozygosity of the parents used to produce the F_2 mapping population. The second map, by Skiba et al. (2004), was constructed using a combination of 47 RAPD, seven sequence-tagged microsatellite site (STMS) and 13 STS/CAPS markers. The map comprised nine linkage groups spanning 803.1 cm, with an average marker distance of 15.8 cm. A backcross mapping population was used in this instance, which simplified the interpretation of dominant marker data. Unfortunately, both maps suffered from the lack of marker saturation of a number of linkage groups, and disappointingly, could not be aligned due to the lack of common anchor markers. More effort is, therefore, clearly needed to develop a comprehensive genetic map for *Lathyrus*.

A growing volume of literature pertaining to the use of cell and tissue culture for improving *Lathyrus* spp. alludes to the emphasis placed by researchers in recent years on developing methods for gene transfer

between members of this genus and with other species in the tribe Vicieae. Additionally, recent promising research by Ochatt et al. (2009) on the use of osmotic and electric shocks to induce androgenesis in a number of *Lathyrus* spp. (*L. sativus*, *L. cicera*, *L. clymenum* and *L. ochrus*) may pave the way for the future application of doubled-haploid technology in breeding programs. The research on the introgression of donor genes through interspecific/intergeneric hybridization in *Lathyrus* was extensively reviewed by Vaz Patto et al. (2006). Most of the attempts to produce interspecific hybrids have failed, with the notable exceptions of, e.g., the successful *L. sativus* × *L. cicera*/*L. amphicarpos* crosses (Yunus and Jackson 1991) and the production of viable hybrids between *L. odoratus* and *L. belinensis* with the aid of embryo rescue techniques (Hammett et al. 1994; Poulter et al. 2003). Protoplast fusion between *L. sativus* and *Pisum sativum* has been attempted by several groups, mainly for the introgression of gene(s) for Ascochyta blight resistance from *Lathyrus* into pea (Vaz Patto et al. 2006). Somatic hybrid calli have been consistently produced (Ochatt et al. 2004); however, regeneration of complete plants from such calli has remained elusive.

6.5 Genomics Resources

The genomics resources for *Lathyrus* are currently limited compared with that of the major crop species but have improved considerably over the past 2 years. At the time of writing, a search of GenBank and GenBank ESTs revealed that there were nearly 18,000 entries for genomic and cDNA sequences for all members of the genus *Lathyrus*, a significant increase over the small number (<50) reported by Skiba et al. (2007). A summary of the available sequence information from the main contributors is available in Table 6.2. Entries by Kenicer et al. (2005) and Oliver (2005, unpublished) were for sequenced fragments of ITS regions and microsatellites, potentially useful for phylogenetic studies and mapping purposes. At present, the only comprehensive cDNA or EST library for *L. sativus* was constructed by Skiba et al. (2005) from *Mycosphaerella pinodes*-challenged leaf and stem tissues. This library, comprising of 818 non-redundant ESTs encompasses the entire spectrum of functional categories with a slight overrepresentation of defense and cell rescue/death/aging ESTs, along with a large number of ESTs (47%) with unknown/unclear function. Of these, the sequences of 177 ESTs, which were used in microarray studies on biotic and abiotic stress resistance in chickpea (*Cicer arietinum*, Coram and Pang 2006; Mantri et al. 2007), were submitted to GenBank (Table 6.2). Although parallel studies were not conducted on *L. sativus*, the existing microarray "The Pulse Chip" and the library will be a particularly useful resource in future studies on biotic and abiotic stress factors in *Lathyrus*. Very recently, Wolfe (2009, unpublished, Table 6.2) submitted a large number of EST sequences of *L. odoratus* to GenBank ESTs, which currently represent the bulk of the genetic sequence information available for *Lathyrus*. Unfortunately, none of the 17,582 sequences were characterized, and to compound the problem, the author acknowledged that the library was contaminated with cDNA from *Danio rerio* (zebrafish). No explanation was provided as to how this occurred or as to the extent of the contamination. For this current publication, one of us (Pang) randomly selected 100 ESTs from Wolfe's (2009) library (using random numbers generated by MS-Excel), BLASTed them against the SWISSProt and SpTrEmBL databases, and finally, assigned each EST into a functional category (Fig. 6.1). The results from this small sample indicated that the zebrafish cDNA contamination was significant (9%);

Table 6.2 *Lathyrus* spp. genetic sequences in GenBank Main and GenBank ESTs as at 25/05/09

Sequence type/gene(s)	Species	Accession numbers	Contributor(s)
EST	*L. sativus*	DY396259–DY396435	Skiba et al. (2005)
EST	*L. odoratus*	GO314003–GO322539	Wolfe (2009, unpublished)
Ribosomal ITS (5.8S, 16S)	53 *Lathyrus* spp.	AY839341–AY839405	Kenicer et al. (2005)
Chloroplast tRNA	53 *Lathyrus* spp.	AY838409–AY839537	Kenicer et al. (2005)
Microsatellite	*L. japonicus*	DQ201753–DQ218041 (with gaps)	Oliver (2005, unpublished)
Convicilin	*L. clymenum*	AM886028–AM886045	Saenz de Miera et al. (2008)

therefore, great caution must be exercised when employing sequence data from this set of *L. odoratus* ESTs. Thirty-seven percent of the ESTs returned either no functional match or were homologous with existing entries representing putative proteins with unknown function. The remaining sequences matched with entries for proteins with known functions (Fig. 6.1), notably from the genera *Medicago*, *Arabidopsis*, *Populus* (poplar), and *Ricinus* (castor oil). The large increase in available EST and genetic marker sequences for *Lathyrus* in the public databases is highly encouraging and will assist in future studies on functional genomics, phylogenetics, and plant breeding. However, the pollution of the largest set of EST sequences (*L. odoratus*) with zebrafish sequences is of concern and must be addressed by the full characterization of the library.

In contrast to the encouraging growth of genomic information discussed earlier, proof-of-function studies of putative *Lathyrus* genes via overexpression or deletion are currently difficult, for a number of reasons. Although an *Agrobacterium*-mediated transformation system for *L. sativus* is available (Barik et al. 2005), it is not widely employed as evidenced by the paucity of publications in this field. Apart from the ODAP mutants discussed previously, there are a number of mutant populations available for studying the effects of gene deletions/silencing. For example, Kumar and Dubey (2003) produced a mutant population of *L. sativus* using gamma rays and a number of chemical mutagens, including EMS (ethylmethane sulphonate) and NMU (N-nitroso-N-methyl urea). The M3 population of 81 individuals displayed significant variation for a number of agronomic characteristics, thus it may be cautiously inferred that this population contained a number of useful mutations. However, for targeting induced local lesions in genome (TILLING) studies, much larger populations will have to be

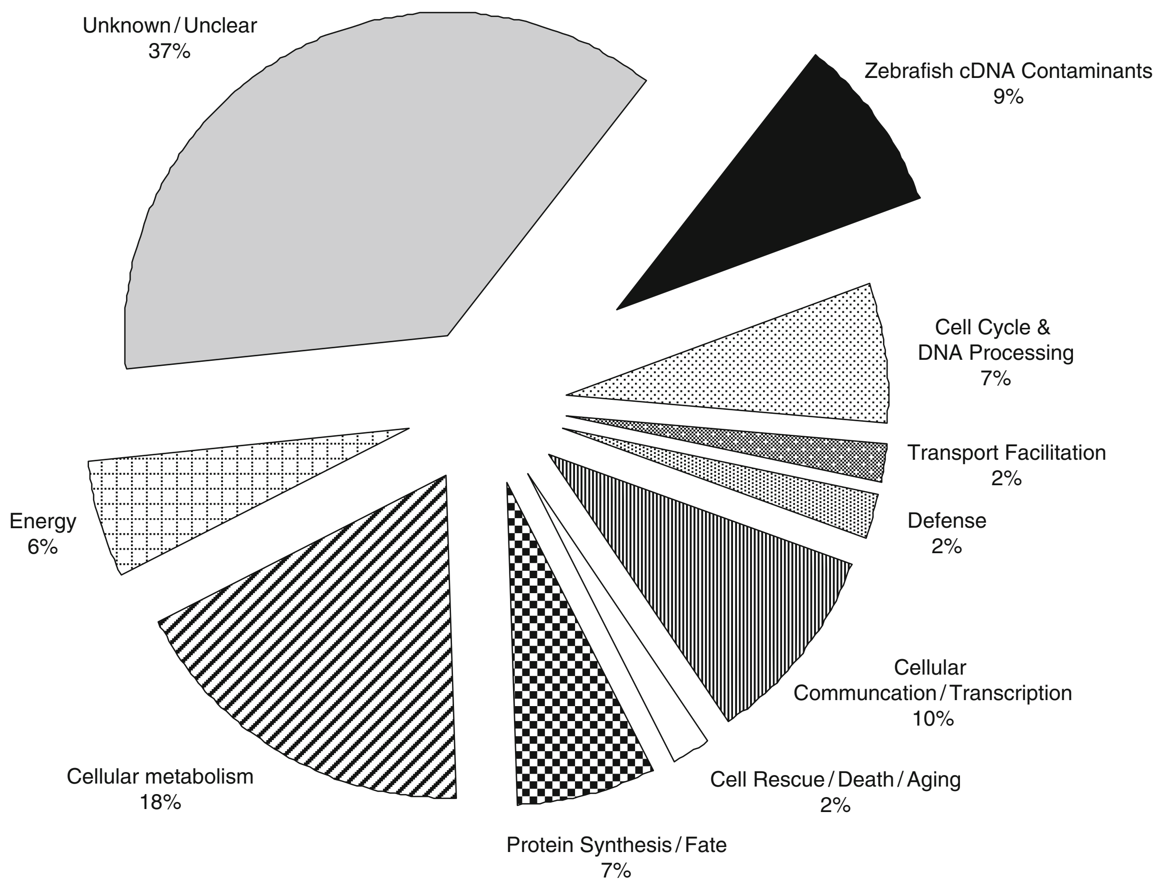


Fig. 6.1 Functional classification of 100 ESTs from *L. odoratus* (Wolfe 2009, unpublished)

constructed. Recently, a promising technique for reverse genetics involving virus-induced gene silencing (VIGS) was developed for *Medicago truncatula* and *L. odorata* (sic) (Grønlund et al. 2008). This technique involved cloning a fragment of the putative gene of interest into the pea early browning virus (PEBV) vector. Upon infection of the plant with the modified virus, the RNAi-based virus defense targets the virus (and the cloned fragment) for degradation. A side effect of this is the post-transcriptional silencing of the target gene, leading to an altered phenotype. Although the technique was successful in producing gene silencing in *L. odorata*, it remains unclear whether it is broadly applicable to all members of *Lathyrus*, especially for the crop species *L. sativus*.

6.6 Recommendations for Future Actions

The genus *Lathyrus* possesses a wealth of genetic variation, but characterization and utilization of the diversity in the main cultivated species (*L. sativus*, *L. cicera* and *L. ochrus*) is still in its early stages, and consequentially, more than 100 wild species have received even less attention. Coordinated efforts to collect and conserve *Lathyrus* crop species have been initiated in the last 10–15 years and have gained momentum with the development of a grasspea conservation strategy as part of the Global Crop Diversity Trust. However, conservation initiatives for the wild *Lathyrus* species need to be expedited before potentially valuable sources of genetic variability are permanently lost.

Progress in crop improvement of *Lathyrus* using traditional and molecular breeding tools has been slow. The main focus of breeding has been to produce low ODAP lines of *L. sativus*, and few reports of advancements in breeding for other traits exist. There is a need to develop a more comprehensive genetic map for *Lathyrus*, with mapped positions of useful genes for marker-assisted breeding. Further, recent progress in protoplast fusion and anther culture require further advancement before these methodologies may be used for *Lathyrus* improvement. The genomics resources of *Lathyrus* are modest compared with that of the major crop species such as wheat, and even with its close relatives, pea and lentil. Much more work is required, particularly in producing EST libraries from specific tissues and organs. As a matter of priority, an EST library constructed from developing pods and seeds should be generated. This is because all of the available ESTs were derived from vegetative rather than reproductive tissues. Characterization of existing ESTs in the databases is also important, as evidenced by the contamination of the main set of *Lathyrus* ESTs with sequences from zebrafish. In terms of plant resources for functional genomic studies, mutant populations are critically needed for gene inactivation/deletion studies. Transformation and VIGS systems for *Lathyrus* appear to be at a nascent stage; therefore, future research should concentrate on the optimization, and wider application of these systems to members of this genus.

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Chapter 7
Lens

Dorin Gupta, Rebecca Ford, and Paul W. J. Taylor

7.1 Introduction

Lentil (*Lens culinaris* Medik. ssp. *culinaris*) is an ancient crop of the Old World, which continues to play a vital role in agriculture. Its primary asset is the ability to produce a high quality protein in drought-prone marginal environments (Cubero 1981). Like other pulses, substantial improvement in the yield potential of lentil has not been achieved due to the lack of a combination of genes for higher productivity, and biotic and abiotic resistance, which are scattered in the cultivated and wild *Lens*.

Wild *Lens* species have emerged as a solution for introducing additional germplasm into cultivated lentil. The wild *Lens* taxa are known to possess resistance against biotic and abiotic stresses (Bayaa and Erskine 1991; Bayaa et al. 1995; Hamdi and Erskine 1996; Hamdi et al. 1996; Gupta and Sharma 2006). Therefore, introgression of desirable genes from related wild *Lens* taxa to the cultivated lentil will help in the flow of useful genes into cultivated lentil and thus including these wild species in the common gene pool (Ladizinsky 1993). The usefulness of these sources to the plant breeder depends on their genetic affinities to the cultigen and availability of gene transfer methods. As far as crossability status of wild *Lens* taxa with cultivated lentil is concerned, *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis* are crossable with cultivated lentil and hence share a common gene pool. Single chromosomal rearrangements may cause partial sterility, but there are still ample opportunities for gene flow and for the utilization of these forms for breeding purposes. Successful hybridization between *L. nigricans*, *L. ervoides*, and cultivated lentil is not yet possible; however, the use of improved techniques of embryo-ovule culture and application of plant growth regulators can help to overcome post-fertilization barriers.

7.2 Basic Botany

7.2.1 Origin and Geographical Distribution

The presumed wild progenitor of the cultivated lentil is *L. culinaris* ssp. *orientalis* (Boiss.) Ponert, which is distributed from Greece in the west to Uzbekistan in the east, and from the Crimean Peninsula in the north to Jordan in the south (Ladizinsky 1979a; Cubero 1981). Lentil (*L. culinaris* ssp. *culinaris*) originated in the Near East (Zohary 1972), where agriculture was adopted as a survival strategy about 10,000 years ago. Afterwards, the crop spread to the Nile and to central Europe. The wide spread use of legumes throughout the Mediterranean basin at the beginning of agriculture is inferred from the distribution of their wild ancestors and the variety of regions in which they have been transformed into domesticated plants. Progressively, local adaptation produced huge diversity within the *L. culinaris* species, resulting in the current pattern of geographic differentiation of landraces (Erskine 1997). Lentil is a widely grown edible grain legume in the semi-arid regions of the world, particularly in the Indian subcontinent and in the dry areas of the Middle East, Canada, southern Europe, and eastern and northern Africa.

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7.2.2 Botany and Morphology

Plants of the genus *Lens* are diploid ($2n = 2x = 14$), which reproduce predominantly by self-pollination. *Lens* is a member of tribe Vicieae, family Fabaceae and subfamily Papilionacea. Besides *Lens*, Vicieae includes the three other genera *Vicia* L. (beans), *Lathyrus* L. (grass pea), and *Pisum* L. (field peas). *Lens* has intermediate ranking between *Vicia* and *Lathyrus*; however, it is more close to *Vicia*. Wild as well as cultivated lentils are annual plants with erect, semi-spreading, and spreading growth habit. The upper leaves have tendrils, which are thin, coiling thread-like extensions that are used for climbing. Flowering starts on the lowest branches and gradually moves up the plant. The small flowers range in color from white or pink to pale blue and lilac. Pods contain one or two small lens-shaped round seeds (Fig. 7.1). However, pod shattering trait is exhibited by wild lentils. *L. culinaris* spp. *culinaris* encompasses two cultivated lentils on the basis of physiomorphological traits, the small-seeded (*microsperma*) and the large-seeded (*macrosperma*) (Barulina 1930). The other subspecies and species are *L. culinaris* ssp. *orientalis* (Boiss.) Ponert, *L. culinaris* ssp. *tomentosus* (Ladz.), *L. culinaris* ssp. *odemensis* Ladz., *L. ervoides* (Brign.) Grande, *L. nigricans* (Bieb.) Godr., and *L. lamottei* Czefr. (Ferguson et al. 2000).



Fig. 7.1 Morphological features of *Lens culinaris* (*http://caliban.mpiz-koeln.mpg.de/~stueber/thome/index.html*)

7.3 Modes of Preservation and Maintenance

Genetic resources offer vital germplasm material for selection and further improvement through breeding to ensure the food security needs of the world's population. The importance of genetic resource conservation arises from the fact that in spite of the vast and continuous demand for new genes, biodiversity is being lost at alarming rates. This biodiversity can be preserved in genebanks (ex situ) and genetic reserves (in situ). Most species show a pattern of genetic variation across their geographic range. *Lens* spp. accessions are mainly maintained and conserved ex situ with little efforts for in situ conservation.

7.3.1 Ex Situ Conservation

Management of ex situ collections requires innovative and adaptive decisions tailored to specific and continuously changing working conditions. Genebanks try to maintain and conserve the original plant diversity from wild relatives and landraces. As far as wild relatives are concerned, they can be utilized for the identification and introgression of novel genes to their cultivated counterparts. International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, has a global mandate for lentil conservation and has the largest collection of lentil germplasm in the world. ICARDA holds 24% of *Lens* germplasm accessions conserved globally, including 583 accessions of wild *Lens*. According to the Genebank Standards published jointly by FAO and IPGRI in 1994, the optimum storage temperature for maximum longevity is −18°C or lower, with the seed moisture content between 3 and 7%, whereas medium term storage and further distribution requires temperatures of 2–5°C. However, drying of seed is recommended at 10–25°C with 10–15% relative humidity (RH) before storage.

A document released in December 2008 as "Global Strategy for the Ex-Situ Conservation of Lentil (*Lens* Miller)" (*http://www.croptrust.org/documents/web/LensStrategy_FINAL_3Dec08.pdf*) provided a blueprint for a more efficient and effective conservation of the *Lens* gene pool throughout the world, including both cultivated and wild species of the genus *Lens*. This strategy identified 43,214 accessions of *Lens* held in collections worldwide, gathered from the questionnaire, meetings, and other data sources. However, *Lens* genetic resources have not been exploited completely, as accession level data is insufficient followed by lack of pre-breeding and other "value-adding" work in genebanks. The strategy report outlined key tasks, such as the completion of necessary regeneration, safety duplication, and storage in long term conditions of unique accessions within the collections, for the formation of a realistic global conservation effort for *Lens* spp.

7.3.2 In Situ Conservation

In situ conservation includes the designation of protected reserve areas in the form of natural parks in which native plants are protected. The park has to be reserved for wild relatives to preserve genetic polymorphism and to provide opportunity for recombination. The wild taxa of lentil are primarily distributed in the Mediterranean basin. The ecological preferences of four wild taxa were described by Ladizinsky (1993). Unfortunately, these genetic resources are being rapidly eroded because of their high palatability to grazing animals, threatening the loss of wild relative genotypes (Ferguson and Erskine 2001). All *Lens* species are self-pollinating with very low outcrossing. *L. culinaris* ssp. *orientalis*, ssp. *tomentosus*, ssp. *odemensis*, and *L. nigricans* are generally found in open or partially shaded habitats, whereas *L. ervoides* usually grows in shady or partially shaded habitats. Ferguson et al. (1998) emphasized that the overall collection priority for *L. culinaris* ssp. *orientalis*, based on the distribution of genetic variation, remains in Southeast Turkey and Northwest Syria, and in South Syria and Jordan. A center of diversity was found to exist in Sweida province, South Syria, for *L. culinaris* ssp. *odemensis* and for *L. ervoides*, along the coastal border region between Syria and Turkey stretching down along the Syrian coast. However, a center of diversity for *L. nigricans* remains in West Turkey. Very few efforts have been made till date for in situ conservation in the areas of highest *Lens* genetic diversity. Some regions of southern Syria identified as genetic reserves for Vicieae species by Maxted (1995) have been selected as genetic reserves by the Syrian Scientific Agricultural Research Commission.

Though some productive steps have been taken for the in situ conservation of wild relatives and landraces of cultivated lentil, more consolidated efforts are required for conserving them for future use.

7.4 Taxonomy

Historical evidence for taxonomic study of *Lens* starts from the work of Alefeld (1866) and is currently based on morphological, crossability behavior, biochemical, and molecular studies.

7.4.1 Classification Based on Morphological Characteristics

Alefeld (1866) classified *Lens* (*L. esculenta* Moench.) into eight subspecies, which included both *orientalis* and *nigricans*. Barulina (1930) made the most detailed study of cultivated lentil on morphological basis. Interestingly, between Alefeld and Barulina, there were no descriptive studies on *Lens* classification. The significant difference between Barulina and Alefeld's classifications was that Barulina selected characters that showed little or no effect of environmental fluctuations for making distinction between subspecies. As far as Alefeld was concerned, he looked for characters such as plant height, vegetation period, color, and pubescence of leaves. Barulina divided cultivated lentil into two subspecies: ssp. *macrosperma* and ssp. *microsperma*. Barulina's work could not be followed by anyone. At present, her classification (ssp. *macrosperma* and *microsperma*) is considered as varieties of ssp. *culinaris*. Barulina did vast studies and also suggested *L. culinaris* ssp. *orientalis* as the wild progenitor of cultivated lentil. Her work was supported by Williams et al. (1974) through detailed morphological comparisons of cultivated lentil and *L. nigricans* and *L. orientalis*.

7.4.2 Classification Supported by Hybridization and Cytogenetic Studies

The use of morphological markers, especially stipule shape for *Lens* classification, was questioned by Ladizinsky (1979a). This trait was considered to be conspicuously plastic and variable. Ladizinsky was the first to perform hybridization experiments and cytogenetic analysis of interspecific crosses between the cultivated lentil and *L. nigricans* and *L. orientalis*. His findings suggested *L. orientalis* to be the wild progenitor of lentil. As a result of cytogenetic and crossability studies, Ladizinsky et al. (1984) recognized two species within the genus *Lens*, *L. culinaris* and *L. nigricans*. *L. culinaris* comprised (1) *L. culinaris* ssp. *culinaris* (cultivated lentil) and (2) *L. orientalis* (wild progenitor of cultivated lentil) (Barulina 1930; Zohary 1972; Williams et al. 1974; Ladizinsky 1979a) and an additional group split from the original *L. nigricans*, *L. odemensis*. *L. odemensis* and *L. culinaris* ssp. *culinaris* were partially interfertile. Ladizinsky et al. (1984) proposed *L. ervoides* as ssp. of *L. nigricans*. However, it was finally concluded that the genus *Lens* consisted of the cultivated lentil, *L. culinaris* ssp. *culinaris*, its wild progenitor *L. orientalis* and five additional wild relatives, viz. *L. odemensis*, *L. ervoides*, *L. nigricans*, *L. tomentosus*, and *L. lamottei*.

7.4.3 Classification Based on DNA Markers

Molecular data using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers of nuclear DNA (Havey and Muehlbauer 1989; Rajora and Mahon 1994, 1995; Abo-Alwafa et al. 1995; Sharma et al. 1995) or cpDNA (Muench et al. 1991; Mayer and Soltis 1994) have supported *L. culinaris* ssp. *culinaris* and *L. orientalis* as one group. The genotype composition of group two and three, however, differed among authors. In addition, Muench et al. (1991) and Abo-Alwafa et al. (1995) found *L. odemensis* in group one. Ahmad and McNeil (1996) reviewed phylogenetic relationships among species of the genus *Lens* by making a comparative study of crossability, RAPD, sodium dedocyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and morphological markers. They concluded that the primary gene pool of cultivated lentil comprised *L. orientalis* and *L. odemensis*, while *L. ervoides* and *L. nigricans* formed a secondary gene pool. Many other workers have supplemented traditional taxonomic methods of identification as

well as studied taxonomic relationships within the genus *Lens* using amplified fragment length polymorphism (AFLP; Sharma et al. 1996) and RAPD (van Oss et al. 1997) markers. Sharma et al. (1995) identified species-diagnostic RAPD markers for *L. odemensis*, *L. ervoides*, and *L. nigricans*. According to Van Oss et al. (1997), the genus *Lens* consisted of seven taxa: *L. culinaris* ssp. *culinaris*, its wild progenitor *L. orientalis*, *L. odemensis*, *L. nigricans*, *L. ervoides*, and two recently recognized species, *L. tomentosus* and *L. lamottei*. However, *L. lamottei*, *L. nigricans, L. odemensis*, and *L. tomentosus* taxa were always questioned because of the lack of clear morphological and molecular markers, which could differentiate between these *Lens* species. Ferguson et al. (2000) took into consideration these aspects and reassessed the taxonomy of *Lens* based on morphological characterization, isozyme electrophoresis, and RAPD techniques. They proposed the most recent classification as given below.

L. culinaris Medik.
- ssp. *culinaris*
- ssp. *orientalis* (Boiss.) Ponert
- ssp. *tomentosus* (Ladiz.) M.E. Ferguson et al.
- ssp. *odemensis* (Ladiz.) M.E. Ferguson et al.

L. ervoides (Brign.) Grande
L. nigricans (M. Bieb.) Godr.
L. lamottei Czefr.

7.5 Classical and Molecular Genetic Studies

The knowledge of inheritance of qualitative and quantitative traits helps in describing new genes, developing linkage maps, and formulating marker-assisted selection strategies. In the present scenario, much of the genetic variability in the cultivated lentil has already been exploited. Of late, the gene pool of cultivated lentil is being enriched by transferring alien chromatin from the wild *Lens* taxa (Goshen et al. 1982; Ahmad et al. 1995; Gupta and Sharma 2007). The introgression from wild to cultivated lentil helps in the flow of useful genes into the cultivated lentil, thus including these wild taxa in the common gene pool. However, the inheritance of many valuable traits in cultivated x wild lentil taxa plays an important role in understanding and confirming the successful introgression of useful characters from wild to cultivated lentil. To date, very little effort has been made on genetic studies and gene mapping for this crop using cultivated x wild lentil populations.

7.5.1 Classical Genetic Studies

Cultivated lentil has been used for the majority of karyotype studies. All *Lens* species have a similar karyotype consisting of one metacentric chromosome with a satellite, three metacentric chromosomes, and three acrocentric chromosomes (Ladizinsky and Abbo 1993). However, Ladizinsky (1979a) concluded from his hybridization experiments and cytogenetic analysis of interspecific hybrids that there were three chromosome interchanges between the cultivated lentil and *L. nigricans* and only one between the cultivated species and *L. culinaris* ssp. *orientalis*. These results suggested the latter species as a wild progenitor of lentil.

In the past, the inheritance pattern of many useful traits was studied in cultivated lentil (Lal and Srivastava 1975; Wilson and Hudson 1978; Vandenberg and Slinkard 1989; Emami and Sharma 1999; Hoque et al. 2002). However, wild x cultivated lentil have not been well exploited for carrying out inheritance studies. Ladizinsky (1979b) reported that erect type of growing habit was under the control of a single dominant gene showing single gene control in the progeny of crosses between *L. culinaris* and *L. orientalis*. Flower color showed segregation for incomplete range of colors, indicating that white was recessive to blue in this study. However, pod dehiscence was dominant over pod indehiscence, and purple epicotyl was monogenically dominant over green epicotyl. Vaillancourt and Slinkard (1992) confirmed growth habit to be monogenic with erect dominant over spreading from *L. odemensis* × *L. culinaris* crosses. However, Emami and Sharma (1999) reported spreading growth habit to be dominant over the erect type in the cultivated lentil. Gupta (2003) studied 12 crosses between cultivated and wild lentil subspecies and found that erect growth habit (*Gh*) was determined by a single gene dominant to semi-spreading growth habit (*gh*). Tendrilled leaf (*Tnl*) was determined by a single gene dominant to tendrilless leaf (*tnl*). Pod shattering (*Pi*) was determined by a single dominant gene to non-shattering pods (*pipi*). Orange cotyledon color (*O*) was determined by a single

gene dominant to yellow cotyledon color (*o*). However, flower color showed digenic (violet vs. white flower color) and monogenic inheritance (violet vs. purple). Violet flower color was dominant to purple and white. The inheritance pattern of these traits helped in the identification of true F_1 plants. However, cotyledon color was reported as the most rapid and valuable genetic marker in hybridization programs to confirm the hybridity of the F_1 seeds.

7.5.2 Molecular Studies

Molecular cytogenetic techniques involving fluorescence in situ hybridization (FISH) of highly repetitive DNA sequences have been used to characterize whole chromosomes or chromosome segments in plant species and to understand the phylogenetic relationships of species (Badaeva et al. 1996; Cuadrado and Jouve 1997; Hizume et al. 2002). Repetitive DNA sequences have been used for chromosome identification and are broadly categorized into dispersed sequences and tandemly organized repeats. Tandemly organized repeats (mostly located at centromeric, telomeric, and (or) interstitial heterochromatin regions of the chromosomes) are useful for chromosome identification (Cuadrado and Schwarzacher 1998; Galasso et al. 2001; Hizume et al. 2002). Very few attempts have been made for karyotype analyses of *Lens* (Slinkard 1985; Galasso et al. 2001; Balyan et al. 2002). Galasso (2003) determined the distribution of the two highly repeated DNA sequences (pLc30 and pLc7) and ribosomal genes for the 18S–5.8S–25S rDNA and the 5S rDNA along chromosomes of all *Lens* species by FISH to provide information on karyotypes, genome organization, and phylogeny. He applied multiple-target FISH on mitotic chromosomes of seven *Lens* taxa and reported that, in general, each species showed a typical FISH karyotype, and a few differences were observed among accessions belonging to the same species, except for the accessions of *L. culinaris* ssp. *odemensis*, which reflected high genetic diversity. The most similar FISH karyotype to the cultivated lentil was that of *L. culinaris* ssp. *orientalis*, whereas *L. nigricans* and *L. tomentosus* were the two species that showed the most divergent FISH patterns compared with all taxa. However, more efforts are required in this field to understand chromosomal relationships between crop plants and their wild relatives, which are important for understanding the evolution of the cultigens and to study their genomic relationships.

7.6 Crop Improvement Through Conventional and Non-conventional Breeding Techniques

Many lentil populations have lost alleles for higher productivity and other economic traits under the present scenario of cultivation. Various workers have reported that there are low amounts of variation in the cultivated lentil germplasm collections (Muench et al. 1991; Ford et al. 1997; Duran et al. 2004). Nevertheless, desirable genes do exist in scattered populations, viz. wild relatives and landraces in different parts of the world. The foremost need, therefore, is to evaluate the wild relatives for different qualitative and quantitative traits besides screening for biotic and abiotic stresses, followed by a hybridization program. *Lens* species within the primary gene pool are readily intercrossed and produce almost fully fertile progenies (Fig. 7.2). Therefore, gene flow between species of the primary gene pool can be accomplished by conventional breeding methods (wide hybridization). However, selection among progenies will help in overcoming any partial fertility. The secondary gene pool contains species that are distant from the cultigens (Fig. 7.2), but it is difficult to obtain gene flow through conventional breeding methods. Therefore, biotechnological methods are required to assist in the introgression of genes between species.

7.6.1 Evaluation of Wild Genetic Resources

7.6.1.1 Agromorphological Traits

Very few attempts have been made to evaluate *Lens* accessions for agromorphological traits. Hamdi and Erskine (1991) evaluated large numbers of accessions from *L. culinaris* ssp. *culinaris*, *L. culinaris* ssp. *orientalis*, *L. culinaris* ssp. *odemensis*, *L. nigricans*, and *L. ervoides* for various morphological, phenological,

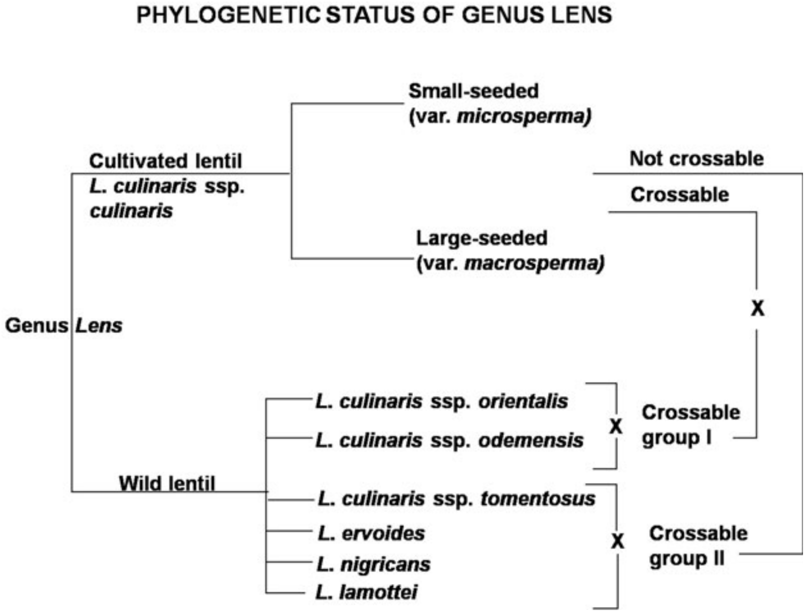


Fig. 7.2 Phylogenetic status of genus *Lens*

and yield related characters and found that some accessions of *L. culinaris* ssp. *orientalis* were early to flower and mature. Ferguson and Robertson (1999) studied morphological and phenological variation in 310 accessions from five *Lens* taxa and reported that certain *L. culinaris* ssp. *orientalis* accessions had substantially more leaves/plant, peduncles/plant, pods/plant, seeds/plant, and leaf area than the two cultivated lentil checks. However, seed yield was greater in both the checks than it was in any of the wild species, which was mainly due to a higher 100-seed weight in the cultigen than the wild taxa. Gupta and Sharma (2006) evaluated wild taxa for morphophysiological traits, and biotic and abiotic stresses, and reported that *L. culinaris* ssp. *orientalis* flowered earlier. However, some were comparable with cultivated genotypes for flowers/peduncle, peduncle length, and plant height. The mean performance for flowers per peduncle, leaflets per leaf, plant height, seeds, and seed yield per plant increased, while there was a decrease in the number of days to flowering and maturity and branches per plant during the evolution of cultivated lentil from the wild *Lens* taxa.

7.6.1.2 Biotic Stresses

The wild *Lens* germplasm possesses novel genes for biotic resistances; however, the gene pool of cultivated lentil is not generally enriched with these identified sources. Bayaa and Erskine (1991) surveyed accessions of *Lens* ssp./sp., comprising *L. culinaris* ssp. *orientalis*, *L. culinaris* ssp. *odemensis*, *L. nigricans*, and *L. ervoides* from nine countries for vascular wilt. Three accessions each of *L. culinaris* ssp. *orientalis* and *L. nigricans* and two accessions of *L. ervoides* exhibited resistance at seeding and adult stage. Bayaa et al. (1994) reported 24 Ascochyta blight-resistant accessions out of 86 *L. culinaris* ssp. *orientalis*, 12 of 35 ssp. *odemensis*, 3 of 35 *L. nigricans*, and 36 of 89 *L. ervoides* accessions screened. Bayaa et al. (1995) also screened 219 accessions of wild *Lens* taxa for resistance to Fusarium wilt at seedling stage. All accessions of *L. culinaris* ssp. *odemensis* were found susceptible, and only three accessions of *L. culinaris* ssp. *orientalis* and one of *L. ervoides* exhibited good resistance. Subsequently, from the 99 accessions of wild *Lens* taxa that were screened by Nasir (1998), 36 of *L. culinaris* ssp. o*rientalis*, four of *L. culinaris* spp. *odemensis*, 14 of *L. ervoides*, and 11 of *L. nigricans* accessions were found resistant. Ahmad et al. (1997) identified sources of resistance to the major diseases of lentil, viz. rust, vascular wilt, and Ascochyta blight in the wild relatives of lentil.

Fusarium wilt (*Fusarium oxysporum* f. sp. *lentis*) is an important fungal disease that limits yields in dry areas. Farmers in Syria, Iraq, and Lebanon have, in the past, relied on a few wilt-resistant cultivars to manage

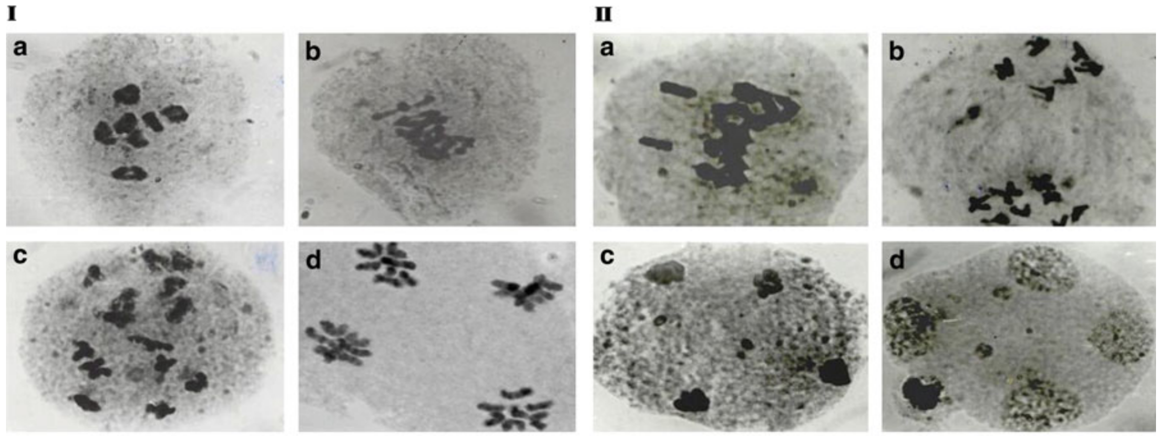
the disease. Recently, researchers from ICARDA screened 3,033 lentil landraces originating from 62 countries, and 247 wild relatives from 14 countries (2000–2007), by growing them on soil infested with Fusarium wilt to identify 28% of landraces resistant to Fusarium wilt. Among the wild relatives, *L. culinaris* ssp. *orientalis* and *L. ervoides* showed good levels of resistance to the disease. These resistant lines were evaluated for agronomic traits, and those with desirable traits were sent to NARS for further evaluation under area specific conditions. Further, results from these areas are being utilized as feedback to improve breeding strategies intended to develop better and well-adapted breeding lines.

7.6.1.3 Abiotic Stresses

Drought and low temperature are the major abiotic stresses causing yield reduction in lentil. However, winter hardiness is a key objective in breeding highland lentil. There are very few reports from wild taxa on screening work for these stresses. Hamdi and Erskine (1996) screened 121 wild *Lens* accessions in the field; however, accessions of cultivated lentil were on an average earliest to flower (86 days). Irrigation prolonged the vegetative growth period in all species. The average seed yield over accessions showed that cultivated lentil produced the highest seed yield and that *L. culinaris* ssp. *orientalis* gave the highest seed and straw yields among wild species. Accessions of *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis* showed an increase over rainfed conditions of 50% in seed yield with irrigation, whereas 156% in case of *L. ervoides*. Greater resistance to cold tolerance has been found in *L. culinaris* ssp. *orientalis* than in the cultivated lentil (Hamdi et al. 1996). In this study, 245 accessions of wild *Lens* taxa, 10 of cultivated lentil, and three accessions of *Vicia montbretii* (syn. *L. montbretii*) were evaluated for winter hardiness in Syria and Turkey. Accessions of *L. culinaris* ssp. *orientalis* exhibited the highest level of winter hardiness, on average, whereas accessions of *L. ervoides* were the most susceptible. Correlations revealed that winter hardiness was concentrated among accessions originating from high elevation areas. More efforts are required to screen wild lentil collections to identify accessions with good levels of tolerance for these stresses. Introgression of such novel genes from wild into cultivated lentil will help to have increased yield with adaptation to stress environments.

7.6.2 Wide Hybridization: Conventional Breeding

Wild relatives of lentil have useful variation for various agromorphological traits along with tolerance to biotic and abiotic stresses. To date, very little research has been carried out to select cultivars from cultivated x wild *Lens* spp. Therefore, there is great need for generation of a wide spectrum of variability through introgression of desirable genes from wild to cultivated lentil, which will help in broadening the genetic base of lentil. Intraspecific hybridization between cultivated and *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis* has been attempted in the past. *L. culinaris* ssp. *orientalis* is readily crossable with the cultivated lentil (Muehlbauer et al. 1989; Vandenberg and Slinkard 1989; Fratini et al. 2004; Gupta and Sharma 2007), although the fertility of the hybrids varies with the chromosome arrangements of the progeny (Ladizinsky 1979a; Ladizinsky et al. 1984). Goshen et al. (1982) reported that chromosome pairing in F_1 plants produced from *L. culinaris* ssp. *culinaris* × *L. nigricans* were highly irregular due to the difference of three translocations between the parental species, and 19% of F_2 plants from this cross showed meiotic stability, full fertility, and chromosomal arrangements similar to that of cultivated lentil. Ladizinsky et al. (1984) developed intersubspecific hybrids between the cultivated lentil and *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis* and concluded that these subspecies shared a common gene pool with the cultivated lentil. Ladizinsky and Abbo (1993) evaluated chromosome pairing in 14 populations of *L. culinaris* ssp. *orientalis* following crosses with the cultivated line LC-2. Bivalent association in the hybrids at meiosis indicated that all 14 populations had a similar chromosome complement of cultivated lentil. Hamdi and Erskine (1994) attempted a few crosses between cultivated lentil and its putative wild progenitor *L. culinaris* ssp. *orientalis*. Ten lines were selected from a bulk segregating population of



I Meiotic analysis of the F_1's of *L. culinaris* ssp. *culinaris* x *L. culinaris* ssp. *orientalis* showing normal (a) diakinesis, (b) metaphase I, (c) anaphase I and (d) telophase II

II Meiotic analysis of the F_1's of *L. culinaris* ssp. *culinaris* x *L. culinaris* ssp. *odomensis* showing (a) two univalents at metaphase I, (b) unequal distribution of chromosomes at anaphase I disjunction, © two micronuclei at telophase II and (d) two micronuclei at tetrad stage

Fig. 7.3 Meiotic stages in lentil hybrids

these crosses. These selections containing genes introgressed from the wild exhibited high average yield among 24 entries of the lentil international yield traits tested in 13–15 countries. Later, Gupta (2003) reported that cultivated lentil × *L. culinaris* ssp. *orientalis* crosses showed substantially higher variability for all the traits than crosses involving cultivated lentil × *L. culinaris* ssp. *odemensis*. Normal meiosis and pollen fertility were observed in the first set of crosses, whereas chromosomal abnormalities and reduced pollen fertility were observed in the second set of crosses (Fig. 7.3). These results indicated that these wild subspecies can be exploited for breeding purpose and their variation can easily be utilized to widen the genetic base of the cultivated lentil.

7.6.3 Biotechnological Techniques: Non-conventional Breeding Approach

7.6.3.1 Tissue Culture

L. culinaris ssp. *orientalis* and *L. culinaris* ssp. *odemensis* are crossable with cultivated lentil and hence share a common gene pool. However, *L. nigricans* and *L. ervoides* are not crossable with cultivated lentil using conventional breeding techniques. The crosses between these two species with cultivated lentil fail due to post fertilization barrier. Ladizinsky et al. (1985) were the first to describe hybrid embryo abortion after interspecific hybridization in lentil. Interspecific hybridization between cultivated lentil and *L. ervoides* and *L. nigricans* resulted in pod development followed by its arrest 10–16 days after pollination and finally the production of shriveled non-viable seeds. This post-fertilization barrier has prevented gene flow from these two wild species into cultivated lentil and utilization of these forms for breeding purposes. Embryo-ovule rescue technique has been used in several crop plants and also reviewed to overcome interspecific fertilization barrier due to hybrid embryo abortion. In lentil, viable and vigorous plants from embryo culture of interspecific crosses between *L. culinaris* ssp. *culinaris* × *L. ervoides* and *L. culinaris* ssp. *culinaris* × *L. nigricans* were obtained on tissue culture medium (MS; Murashige and Skoog 1962) supplemented with plant growth regulators (Cohen et al. 1984; Ladizinsky et al. 1985).

Later, Abbo and Ladizinsky (1991) observed hybrid embryo abortion at about 14 days after pollination (DAP) in crosses between *L. culinaris* ssp.

culinaris and *L. ervoides*, regardless of the direction of the cross, and in crosses between *L. culinaris* ssp. *culinaris* and specific accessions of its wild progenitor, *L. culinaris* ssp. *orientalis*. At 14 DAP, the hybrid embryos had smaller cotyledons, initiation of shoot and root primordia, and remnants of the endosperm. They suggested that the use of embryo culture technique would produce vegetatively normal plants that were partially fertile. Plant growth regulators were also helpful for the production of successful interspecific hybrids when they were applied after fertilization to harvested healthy seed. Ahmad et al. (1995) attempted 30 interspecific crosses among cultivated lentil and four wild species (*L. culinaris* ssp. *orientalis*, *L. culinaris* ssp. *odemensis*, *L. ervoides*, and *L. nigricans*) and obtained viable hybrids through artificial application of gibberellic acid (GA_3) after fertilization for the normal growth of the hybrid embryo. *L. culinaris* ssp. *culinaris* × *L. nigricans* crosses showed 100% successful hybridization, followed by *L. culinaris* ssp. *culinaris* × *L. orientalis* (66% success) and 50% success for rest of the crosses. However, all of them could not be carried to harvest. Hybrid embryos cultured on artificial medium showed no growth and died after 3 weeks. Gupta and Sharma (2005) used a two step in vitro method of embryo-ovule rescue to overcome the post-fertilization interspecific barrier. The hybrid embryo differentiated into single and multiple shoots, but it could not differentiate into roots. The medium was standardized for ovule and embryo rescue and its differentiation into shoots.

Fratini and Ruiz (2006) conducted four different hybridization experiments to obtain interspecific hybrids. In the first two sets, with only pollination and pollination with the addition of gibberellic acid after fertilization, no lentil hybrids were recovered. A single interspecific hybrid with *L. culinaris* ssp. *odemensis* was obtained in the third experiment using the embryo rescue protocol of Cohen et al. (1984). However, in the last experiment, with their new protocol, ovule-embryos of 18 DAP were cultured on MS salts with 1% sucrose and 1 μM indole acetic acid (IAA) + 0.8 μM Kinetin (KN); after 2 weeks, embryos were released from the ovular integuments. Afterwards, the embryos were transferred to test tubes containing the same medium, and plantlets were obtained 1 month later. Of 1,707 pollinations, six interspecific hybrids with *L. odemensis*, two with *L. nigricans*, and one with *L. ervoides* were recovered.

Successful in vitro multiplication of an intraspecific and an interspecific (Alpo × *L. culinaris* ssp. *odemensis*) lentil hybrid in three consecutive micropropagation cycles was reported by Fratini and Ruiz (2007), to increase the production of F_2 seeds. A total of 982 F_2 seeds were produced in the experiment with Alpo × *L. culinaris* ssp. *odemensis*, consisting of 334 F_2 seeds of the original hybrid and 648 F_2 seeds produced by the 12 plants cloned. Consequently, F_2 seed production was increased threefold over the original hybrid (194%). These results suggested that micropropagation of hybrids was an important tool to acquire large F_1 populations from a single individual that would lead to sufficient production of F_2 seeds useful for genetic studies and breeding.

7.6.3.2 Molecular Markers

Wild *Lens* relatives have shown vast potential to increase the variation of the cultivated gene pool. However, introgression of desirable genes from wild to cultivated genome will also transfer segments of chromosomes of various sizes, which may have deleterious genetic material that requires elimination through backcrossing. Linkage maps and molecular marker systems are important tools for tagging and transfer genes of interest without bringing the closely linked undesired chromosome sequences. Tanksley et al. (1989) demonstrated that conventional backcross breeding will recover 99% recurrent genome in 6.5 generations, whereas marker-assisted backcrossing can recover 100% recurrent genome in just three generations. They also illustrated that conventional backcrossing would take 100 backcrosses to reduce linkage drag to 1 cm; however, it requires two backcrosses by marker-assisted backcrossing in the context of a highly saturated linkage map. Molecular markers are very useful when there is a well-developed genetic linkage map. Nine linkage groups of *Lens* were proposed by Havey and Muehlbauer (1989) and Muehlbauer et al. (1989) using the mapping population derived from an interspecific cross between *L. culinaris* ssp. *culinaris* and *L. culinaris* ssp. *orientalis*. Weeden et al. (1992) developed eleven linkage groups covering 560 cm distance using 64 markers. Tahir et al. (1993) compared the data from different studies and proposed 10 tentative linkage groups. The maps that have been developed for lentil are

particularly useful for gene introgression from wild relatives, since these maps have been developed from interspecific crosses of the cultivated lentil and its presumed progenitor.

Until recently, the genetic maps of this species consisted of a relatively small number of markers, which was further enriched by Eujayl et al. (1998), Rubeena et al. (2003), Duran et al. (2004), and Hamwieh et al. (2005) and covered a small portion of the lentil genome. A recent study (Phan et al. 2007) showed a clear evidence of a simple and direct macrosyntenic relationship between lentil and *Medicago truncatula*. This can, however, be resolved to an extent with the available framework of lentil genetic map and the possible use of genomic information available from the model legume species. Mapping and dissection of quantitative trait loci (QTL) can be aided greatly by using molecular markers. Comparative genomics has become an important strategy for extending genetic information from model species to more genetically complex species (Paterson et al. 2005). Studies have demonstrated that comparative genome analysis can reveal genetic conservation among genomes of closely related species and can greatly facilitate gene discovery (Sorrells et al. 2003; Hartmann et al. 2006; Jaiswal et al. 2006). The conserved regions, the so-called syntenic or orthologous regions, have colinear gene contents when compared either genetically or physically (Phan et al. 2007). Therefore, the development and integration of markers in a lentil genetic map using model genome information will increase its repertoire for mapping of genes and dissecting QTL associated with important plant traits.

7.7 Concluding Remarks

Global agriculture in the twenty-first century is facing unparalleled challenges. Despite an era of modern techniques and technologies for crop improvement, there is still the need to better integrate the knowledge acquired through conventional and non-conventional breeding approaches. A narrow genetic variability in cultivated lentil has hampered the enormous efforts put forth for improving the yield and quality of its produce. Wild *Lens* taxa have shown vast potential for improving and increasing the produce of cultivated lentil. However, conventional crop improvement approaches have not been able to successfully exploit the species belonging to the secondary gene pool (*L. nigricans* and *L. ervoides*) due to post-fertilization barriers. Tissue culture techniques, such as embryo rescue, have been used to overcome post-fertilization barriers. Other advanced techniques, such as molecular karyotyping, doubled haploid production, and regeneration from protoplast culture, are potential areas for widening the gene pool of cultivated lentil through inclusion of these wild relatives into the common gene pool. Molecular marker-facilitated introgression is an emerging breeding tool, which can be used effectively in the selection of progenies from interspecific hybridization. Use of PCR-based molecular markers, such as expressed sequence tags (EST), sequence related amplified polymorphism (SRAP), and single nucleotide polymorphism (SNP), which are sequences of DNA generated from random or specific primers (oligonucleotides) via PCR from same or related genera followed by marker-assisted selection, will bring research for this important legume to the front line. Moreover, with the exploitation of innovative gene expression tools, such as microarray technique, the whole genome of lentil can be studied and genes of interest identified from thousands of genes expressed in response to a particular abiotic or biotic stress simultaneously, regardless of whether they are cultivated or wild lentil species.

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Chapter 8
Lotus

Shusei Sato and Satoshi Tabata

8.1 Introduction

8.1.1 Botanical Characteristics of the Lotus *Species*

The genus *Lotus* comprises more than 180 species, both annuals and perennials species, adapted to a wide range of ecological habitats. A large number of *Lotus* species provides genetic diversity, which is essential in breeding for a variety of agronomic traits, resulting in plants capable of adapting to different environmental conditions. Agronomical *Lotus* species are classified in the subgenus *Edentolotus,* section *Xantolotus* (Izaguirre and Beyhaut 1998; Arambarri 1999). Four species have been domesticated and subjected to breeding to improve pastures and hay quality: *L. corniculatus* (bird's foot trefoil), *L. glaber* (narrow-leaf trefoil), *L. subbiflorus* (hairy bird's-foot trefoil), and *L. uliginosus* (greater lotus), (Papadopoulos and Kelman 1999). Among these, *L. corniculatus* is considered to be the species with the greatest agricultural importance and the widest distribution. Comprehensive geographical, morphological, and genetic analyses have been carried out to deduce the ancestry of this species. *L. corniculatus* essentially has an allotetraploid genome, and four existing species with diploid genomes ($2n = 12$), *L. alpinus*, *L. japonicus*, *L. tenuis*, *L. uliginosus*, and *L. alpinus* [*L. alpinus* also possesses tetraploid ($2n = 24$) and hexaploid ($2n = 36$) genomes], have been speculated as possible ancestors to *L. corniculatus* (Grant and Small 1995).

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8.1.2 Germplasm Banks

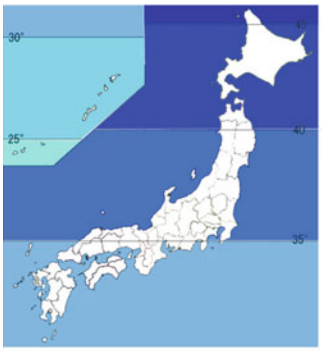
8.1.2.1 National Plant Germplasm System (NPGS, USA)

Though the US *Lotus* collection has been housed at the Northeast Regional Plant Introduction Station (NERPIS) in Geneva, New York, the forage crop collections including *Lotus* were transferred from Geneva, NY, to the Western Regional Plant Introduction Station (WRPIS) in Pullman, WA. Seed from the *Lotus* collection will be housed and distributed from the Pullman, WA facility, but the collection will be managed and regenerated in Prosser, WA (*http://www.ars.usda.gov/Main/site_main.htm?modecode=53-48-15-00*). Currently, the collection has 900 accessions, which represent 60 species from 65 countries, including Italy, Russia, Armenia, Turkmenistan, and Tajikistan. The seeds of a total of 734 accessions are freely available for distribution for research purposes and can be requested using the Germplasm Resources Information Network (GRIN) (*http://www.ars-grin.gov/npgs*; Green 2005).

8.1.2.2 National BioResource Project (NBRP, Japan)

In 2004, *L. japonicus* was selected as a target by the National BioResource Project, Japan, whose aim is to support the development of legume researches by providing access to their research material. The seeds of experimental lines, 108 wild accessions collected from Hokkaido to Okinawa in Japan (Fig. 8.1), 205 recombinant inbred lines (RILs) generated between the Gifu and Miyakojima accessions, 171

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Wild Accession Strains List

About Wild Accessions

Search Keyword Latitude : 40 <= 45

Acc.No.	Location of Seed Collecting			Tribal name	Date	Depositor	Collector	Request	Latitude
	Prefecture	Town	Location						
MG-2	Hokkaido	Taiki		Taiki1	1993.1	T. Sato	T. Sato M. Kawaguchi	Out of stock	42 ° 34 ' 19 " N
MG-23	Aomori	Aomori		Aomori		T. Nemoto	T. Nemoto	Available	40 ° 49 ' 25 " N
MG-34	Hokkaido	Toyokoro	Lake Yudo	Toyokoro	1980.9.19	T. Shimada	T. Shimada	Available	42 ° 36 ' 51 " N
MG-35	Hokkaido	Noboribetsu	Sea side	Noboribetsu	1979.8	T. Shimada	T. Shimada	Available	42 ° 26 ' 44 " N
MG-36	Hokkaido	Oshamambe	Sae side Toyotsu	Oshyamanbe1	1979.8	T. Shimada	T. Shimada	Available	42 ° 24 ' 34 " N
MG-38	Hokkaido	Mori	Karasu Yamazaki River	Mori1	1979.8	T. Shimada	T. Shimada	Out of stock	42 ° 6 ' 29 " N
MG-39	Aomori	Hiranai	Mouth of Shimizu River	Hiranai	1979.8	T. Shimada	T. Shimada	Available	40 ° 56 ' 39 " N
MG-40	Aomori	Towada	Okuirise River	Towada	1979.8	T. Shimada	T. Shimada	Available	40 ° 35 ' 13 " N
MG-90	Hokkaido	Sapporo	NARCH	Sapporo	1981.8.7	NARCHR	NARCHR	Available	43 ° 0 ' 16 " N
MG-91	Hokkaido	Ono	Ono River	Ono	1981.8.20	NARCHR	NARCHR	Available	41 ° 53 ' 44 " N
MG-92	Hokkaido	Tokoro	Wakka	Tokoro	1981.8.20	NARCHR	NARCHR	Available	44 ° 9 ' 36 " N
MG-93	Hokkaido	Mori	Mt. Komagatake	Komagatake	1981.8.5	NARCHR	NARCHR	Available	42 ° 2 ' 1 " N
MG-94	Hokkaido	Date	Kogane Sea Side	Date	1981.8.6	NARCHR	NARCHR	Available	42 ° 25 ' 7 " N
MG-95	Hokkaido	Shiraoi	Kojuhama Sea Side	Shiraoi	1981.8.6	NARCHR	NARCHR	Available	42 ° 28 ' 31 " N
MG-96	Hokkaido	Tomakomai	Nisikioka	Tomakomai	1981.8.18	NARCHR	NARCHR	Available	42 ° 36 ' 10 " N
MG-97	Hokkaido	Oshamambe	Kun-nui	Oshyamanbe2	1981.8.19	NARCHR	NARCHR	Available	42 ° 26 ' 59 " N
MG-98	Hokkaido	Nanae	Shirotai Ranch	Nanae	1981.8.19	NARCHR	NARCHR	Available	41 ° 55 ' 15 " N
MG-99	Hokkaido	Hakodate	Mt. Hakodate	Hakodate	1981.8.20	NARCHR	NARCHR	Available	41 ° 45 ' 25 " N
MG-100	Hokkaido	Yakumo	Higashino	Yakumo	1981.8.20	NARCHR	NARCHR	Available	42 ° 13 ' 44 " N
MG-101	Hokkaido	Mori	Akai River	Mori2	1981.8.20	NARCHR	NARCHR	Available	42 ° 0 ' 11 " N

Fig. 8.1 List of *L. japonicus* wild accessions collected by the National BioResource Project (NBRP). Among the wild accessions in the NBRP, information is listed for the accessions collected from the area between latitudes 40 and 45°N (*left-hand figure*)

ethylmethane sulfonate (EMS) mutants, and genomic resources such as BAC/TAC genomic and cDNA libraries are distributed through the Legume Base database (Table 8.1) (Isobe and Akashi 2004). Some phenotypic data evaluated at Miyazaki (N. L. 31°, Long. 131°E) and Sapporo (N. L. 43°, Long. 141°E) are also provided through the database.

8.2 Diploid *Lotus* Species for Genomics

8.2.1 Lotus japonicus

L. japonicus (Regel) Larsen was proposed as a model legume for genetics and genomics in 1992 because of its various characteristics such as small genome size, small plant size, large and abundant flowers, easy hand pollination, high seed production, short generation time, easy cultivation, and its amenity to plant transformation and regeneration from tissue culture (Handberg and Stougaard 1992). Since then, a set of genetic resources and tools has been rapidly developed. These have included ecotypes, mutant lines, genetic maps, recombinant inbred lines (RILs), a transformation technology, expressed sequence tags (ESTs), and a draft sequence covering the whole genome. Accordingly, the *L. japonicus* research has been drastically accelerated and has greatly contributed to the understanding of the symbiotic processes with both Rhizobium and mycorrhizal fungi. This has allowed the cloning of several key genes involved in the processes of symbiosis with both microbes.

For molecular genetics and genomics in *Lotus* species, two accessions of *L. japonicus*, Gifu B-129 and Miyakojima MG-20, have been mainly used. And very recently, another wild accession, *L. bruttii* B-303 derived from West Pakistan, is attracting attention

Table 8.1 Web databases for *Lotus* species resources

Site name	Contents	URL
Legume base	Detailed information and request forms for experimental strains, wild accessions (108 lines), B-129 × MG-20 RILs, genome clones, and cDNA clones	*http://www.legumebase.agr.miyazaki-u.ac.jp/index.jsp*
miyakogusa.jp	Information on genome sequences, predicted genes, genetic maps, and DNA markers	*http://www.kazusa.or.jp/lotus/*
Lotus Tilling	Detailed information on *Lotus* TILLING system and instructions for screening requests	*http://www.lotusjaponicus.org/tillingpages/homepage.htm*
Lotus japonicus mutant finder	Information from the screening of M_2 families of *L. japonicus* generated by EMS mutagenesis. Search system by characteristics is available	*http://data.jic.bbsrc.ac.uk/cgi-bin/lotusjaponicus/*
DFCI *L. japonicus* gene index	Information on TC and singleton EST sequences	*http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=l_japonicus*
Lotus Newsletter	Sharing information for the *Lotus* species research community	*http://www.inia.org.uy/sitios/lnl/*

because of its early flowering characteristics and moderate genetic diversity, which enable design of DNA markers against *L. japonicus*, Gifu B-129, and Miyakojima MG-20.

8.2.1.1 Gifu B-129

The original source of B-129 was collected in the Gifu prefecture of Japan in the mid-1900s by Isao Hirayoshi of Kyoto University. An inbred line of B-129 was established by multiplication of this plant, and the S_9 generation constituted the Gifu B-129-S9 germplasm. In 1992, Handberg and Stougaard demonstrated that *L. japonicus* B-129-S9 is suitable for genetic and molecular biology research because (1) it is a diploid ($2n = 2x = 12$), perennial, autogamous legume with good seed set, and has a sexual regeneration time of approximately three months; (2) it has a relatively small haploid genome size, estimated at 0.5 pg per haploid complement; (3) it is susceptible to *Agrobacterium tumefaciens*, and transgenic plants can be regenerated. Stable transformation followed by regeneration has been demonstrated (Handberg and Stougaard 1992; Thykjaer et al. 1995).

The Gifu B-129-S9 plant grows with multiple branches up to 30 cm long and is cross-fertile, making classical genetic studies possible. Seed of this release has been deposited in the National Plant Germplasm System through the USDA-ARS Regional Plant Introduction Station, Pullman, WA 99164-6402, and can be distributed for research purposes as PI 591056, as described above.

8.2.1.2 Miyakojima MG-20

L. japonicus Gifu B-129 is a long-day plant and requires intense light for continuous flowering. Therefore, genetic analysis of this plant is often conducted in a greenhouse equipped with supplementary light. In order to develop a legume model system for legume genetics, as has been done with *Arabidopsis thaliana,* early-flowering accessions, suitable for indoor handling, were searched for throughout Japan. As a result, a plant that grows wild in Miyakojima, one of the southernmost islands in the Japanese archipelago, was collected as the earliest-flowering accession by Masayoshi Kawaguchi of the University of Tokyo. The accession was named as Miyakojima MG-20, and after seven rounds of self-pollination, the Miyakojima MG-20-S7 germplasm was established in 2000 (Kawaguchi 2000). This accession has predominantly been used for development of genomic resources such as cDNA and genomic libraries, ESTs, whole-genome sequences, high-resolution genetic maps, and ion-beam mutants (Hayashi et al. 2001; Kawaguchi et al. 2001; Sato et al. 2001; Asamizu et al. 2004). The seeds of Miyakojima MG-20 can be obtained through the Legume Base (Table 8.1).

8.2.2 L. bruttii

L. burttii was collected from the banks of the Kabul River in Pakistan, and the germplasm *L. burttii* B-303-S9 was established in 2005 from the S_9 seed after

self-pollination in a greenhouse or a growth cabinet without pollinators (Kawaguchi et al. 2005). The most notable characteristic of *L. burttii* is its early flowering time, 1–2 weeks earlier than that of *L. japonicus* Miyakojima MG-20. The *L. burttii* B-303 plant is relatively small in size compared to Gifu B-129 and Miyakojima MG-20. The flowers of *L. burttii* are pale yellow and turn red during maturation. Pods rarely shatter even under dry conditions, whereas those of *L. japonicus* shatter very easily after pod maturation. The possession of such traits, ideally suited to a model system, means that *L. burttii* is expected to contribute to the molecular genetics of legumes. In addition, *L. burttii* has a higher level of DNA polymorphism compared to Gifu B-129 and Miyakojima MG-20, enabling design of codominant DNA markers. Hence, molecular genetics using *L. burttii* will make a contribution to isolation and functional characterization of legume genes in the future.

8.3 Genetic Maps of *Lotus* Species

In order to increase the efficiency of genetic analysis in the model legume *L. japonicus*, two high-density genetic linkage maps have been developed from an intraspecific cross between the two ecotypes *L. japonicus* Gifu B-129 and *L. japonicus* Miyakojima MG-20 (Hayashi et al. 2001), and an interspecific cross between *L. japonicus* Gifu B-129 and *L. filicaulis* (Sandal et al. 2002). For faster mapping of new loci, a selection of reliable markers spread over the chromosome arms provides a common framework for more efficient identification of new alleles. Combination of this mapping effort with the genome sequencing of *L. japonicus* has led to more than 2,000 genomic clones being anchored to the genetic linkage map through molecular markers (Hayashi et al. 2001; Sato et al. 2001, 2008). Based on a set of anchor markers selected from the markers generated through the sequencing project, the two genetic linkage maps were aligned, and regions of linkage groups, where genetic resolution is obtained preferentially using one or the other parental combination, have been highlighted (Sandal et al. 2006). A third genetic map was established from a cross between *L. japonicus* Gifu B-129 and *L. burttii* to obtain better genetic resolution in regions showing suppression of recombination or distorted segregation in one or both of the above-mentioned maps (Kawaguchi et al. 2005). Thus, three different diploid species, *L. japonicus*, *L. filicaulis*, and *L. burttii* form a *Lotus* molecular genetic triangle.

Additional genetic resolution and stabilized mapping populations, RILs, are important resources for the mapping of single genes as well as of quantitative trait loci (QTLs). From the two original F_2 mapping populations, RILs have been developed to homozygosity by single-seed descent to the S_8 generation. To date, more than 100 RILs have been developed for the cross population between *L. japonicus* Gifu B-129 and *L. filicaulis* (Sandal et al. 2006), and a total of 205 RILs have been developed for the cross between *L. japonicus* Gifu B-129 and Miyakojima MG-20 (Sato et al. 2001). An initial map of this mapping population can be seen at *http://www.kazusa.or.jp/lotus/RIline/RI_map.html*; and the seeds of these lines can be obtained through the National BioResource Project of Japan (*http://www.legumebase.agr.miyazaki-u.ac.jp/*). RILs are currently being propagated from the cross between *L. japonicus* Gifu B-129 and the *L. burttii* population, adding further genetic and biological variability. Altogether, these research efforts have established a common genetic resource for *Lotus* species.

For the agronomical *Lotus* species, the first genetic linkage map for *L. corniculatus* has been developed using a combination of restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), intersimple sequence repeat (ISSR), sequence tagged site (STS), and isozyme markers (Fjellstrom et al. 2003). This linkage map will help to establish a basis for marker-assisted selection for the improvement of desirable characteristics in agronomical *Lotus* species.

8.4 Role in Crop Improvement Through Traditional and Advanced Tools

8.4.1 QTL Analysis of Agronomically Important Traits

Many agronomically important traits are controlled by plural genes known as quantitative trait loci (QTLs). Recent advances in genomic research on the model plants have made it possible to examine the naturally

occurring allelic variation for agronomically important traits (Varshney et al. 2006). Therefore, model plants have been expected to play a role as a platform for exploring QTLs of useful traits. In order to apply further the knowledge obtained on *L. japonicus* to breeding legume crops, it is necessary to identify genomic regions related to agriculturally important traits. A QTL analysis for agronomic traits in *L. japonicus* has been performed using the population of RILs derived from the cross between Gifu B-129 and Miyakojima MG-20. Thirteen agronomic traits evaluated over 2 years included flowering traits (flowering time and degree); traits of vegetative parts (plant height, stem thickness, leaf length, leaf width, plant regrowth, plant type, and stem color); and reproductive traits (pod length, pod width, seeds per pod, and seed weight). A total of forty QTLs were detected, which explained 5.1–69% of the total variation. Some of them are mapped to the positions corresponding to the QTL positions of soybean, pea, and chickpea in the macrosyntenic relationship (Gondo et al. 2007). The information on QTLs accumulated for the model legume *L. japonicus* could be applied in developing a breeding program for agronomically important *Lotus* species as well as for legume crops through genomic synteny.

8.4.2 Breeding of Lotus Species

Improvements in *Lotus* species aim to make them more persistent, reliable, and productive. A large genetic variation exists in *Lotus* species, providing scope for further genetic improvement. Variation for agronomic and herbage quality characteristics including plant decumbency, winter injury, herbage dry mass, and in vitro digestible dry matter exists among different accessions.

Improving persistence is a complex task because plant death or stand loss results from the combined effects of disease susceptibility, adverse environment, low reproduction rate, and limited genetic variation. The persistence of a *Lotus* stand can vary depending upon the amount of disease, the level of natural reseeding, and the type of cultivar. Characteristics of specific interest to improve the persistence of *Lotus* species include rhizomatous growth habit, disease resistance, condensed tannins, and hydrocyanide content. Breeding for improved cultivars in *Lotus* species has been carried out by phenotypic recurrent selection for general and specific combining ability to identify desired genetic combinations. The transfer of rhizomatous growth from the putative *L. corniculatus* obtained from Morocco to the domesticated *L. corniculatus* has been developed with the purpose of increasing long term persistence, and evaluation studies are being carried out in USA in cooperation with INIA-Uruguay. In Australia, the improvement of *L. uliginosus* is focusing on optimizing the levels of condensed tannins to provide bloat protection. In Argentina, selection among naturalized ecotypes of *L. glaber* has been the basis of cultivar development for alkaline and saline soils of the Pampas. The breeding of cultivars with improved disease resistance, vigor, ability to reseed, or to spread by rhizomes should make these *Lotus* species more reliable and productive. A list of *Lotus* cultivars has been published in the *Lotus Newsletter* (Grant 2004).

The knowledge derived from the model legume, *L. japonicus*, will be transferred to the agronomical *Lotus* species. The slow progress observed in *Lotus* cultivar improvement could be explained by the cross-pollination of species and the fact that some of them are polyploidy. These features are not present *in L. japonicus*. One of the tasks in attaining species improvement will be to determine the relationship between the agronomical species and the origin of *L. corniculatus*. Biochemical and genetic evidences indicate that *L. japonicus*, *L. glaber*, and *L. uliginosus* together with *L. alpinus* are likely to be the ancestors of *L. corniculatus* (Grant and Small 1995).

8.4.3 Genetic Transformation and Somaclonal Variation

One of the important characteristics of *Lotus* species is the feasibility of regeneration of plants in culture, which makes them amenable to genetic transformation by *A. tumefaciens* and *A. rhizogenes* (Armstead and Webb 1987; Handberg and Stougaard 1992). This advantage has been largely applied to basic studies, such as complementation of mutants and metabolic pathway control using expression of transgenes, although no cultivar has been released through genetic manipulation so far.

Along with the effects of the introduced genes themselves, genetic transformation process has significant potential for generating new genetic variation known as somaclonal variation. In *L. japonicus*, mutant lines were generated from regenerated plants, and these lines were used for isolation of nodulation mutants (Schauser et al. 1999). During the detailed analysis of these mutant lines, a retrotransposon named LORE1 was identified as one of the causes of somaclonal variation (Madsen et al. 2005). In the agronomical *Lotus* species, somaclonal variation has been found in *L. corniculatus* regenerated from cell cultures (Vessabutr and Grant 1995). Regenerated plants from *L. corniculatus* have proved to be suitable for the evaluation of somaclonal variation in morphological and agronomical traits.

8.5 Genomics Resources for *Lotus* Species

Genomic resources of *Lotus* species have been accumulated from the model legume, *L. japonicus*.

8.5.1 EST Analysis

Expressed sequence tag (EST) analysis is performed to generate a catalog of expressed portions of the genome by developing anonymous partial cDNA sequences. Currently, nearly 150,000 ESTs have been deposited in public databases (Szczyglowski et al. 1997; Endo et al. 2000; Asamizu et al. 2004; Colebatch et al. 2004) and assembled into 15,472 tentative consensus (TC) sequences (and 18,713 singleton ESTs) in the *L. japonicus* gene index database (Table 8.1). These ESTs as well as the corresponding cDNA clones are providing valuable information and material resources for the functional analysis of individual genes and of the genome.

8.5.2 Genome Sequences

Genome sequences, which complement the ESTs, provide a global picture of the genetic information carried by living organisms. To understand the genetic systems carried by leguminous plants, large scale genome sequencing of the *L. japonicus* accession, Miyakojima MG-20, is in progress (Sato et al. 2001, 2008). To sequence the *L. japonicus* genome, two parallel approaches are being used: (1) the clone-by-clone method and (2) shotgun sequencing of selected genomic regions enriched for the gene space. Both have been very effective in supporting collaborative mapping and map-based cloning experiments, where many of the mechanisms governing legume biology, including root symbioses, have been uncovered.

An insight into the genome of *L. japonicus* has been reported based on the draft genome structure of 315.1 Mb, which covers 67% of the total genome's physical length and is presumed to represent approximately 91.3% of the *L. japonicus* gene space, as predicted from the TC sequences available (Sato et al. 2008). In total, 41% of the draft genomic sequence was positioned onto the *L. japonicus* genetic map, and this percentage is likely to increase substantially when the ongoing single nucleotide polymorphism (SNP) detection and mapping is finalized in the near future.

A total of 30,799 potential protein-encoding genes (excluding those for transposons/retrotranspons) have been assigned to the analyzed genome sequence. Comparative analysis of these genes has revealed the expansion of several functional domains and gene families that are characteristic of *L. japonicus*. Detailed information on these predicted genes as well as information on the nucleotide sequences of pseudomolecules and DNA markers is available through the online database "*miyakogusa.jp*" (Table 8.1).

8.5.3 TILLING

TILLING (targeting-induced local lesions in genomes) is a high-throughput, reverse genetic approach used to identify individuals with point mutations in genes of interest from large EMS-mutagenized populations (McCallum et al. 2000). TILLING relies on the ability of the CEL1 endonuclease to detect mismatches between normal and mutant DNA strands when they are annealed. Several populations of *L. japonicus* Gifu B-129 are accessible for TILLING. One of these is a general-purpose TILLING population that is biased against the occurrence of severe developmental phenotypes and comprises about 5,000

plants, each representing an independent family (Perry et al. 2003). The average mutation frequency of six mutations per 2.3 Mb of sequence suggests that this population bears approximately 1,300 mutations per genome, which is similar to the value reported for the *Arabidopsis* TILLING facility. Smaller subpopulations of several hundred plants are composed of M_2 siblings defective in root nodule symbiosis and starch synthesis. Furthermore, to facilitate similar TILLING approaches for other traits, seeds from a variety of developmental mutants have been collected, and trait-specific populations for traits such as altered leaf or flower morphology can be assembled. Phenotypic descriptions and photographs of the various mutants have been entered onto a web-accessible database for people to browse and order mutants (Table 8.1). Details for accessing the *Lotus* TILLING facility are available through the website *http://www.lotusjaponicus.org* (Table 8.1).

8.5.4 Transcriptomics

Two approaches have been taken to measure transcript levels of *L. japonicus*, including serial analysis of gene expression (SAGE) and analysis using DNA arrays.

SAGE is a sequence-based technology in which a genome-wide transcript analysis can be conducted. SAGE generates short cDNA fragments, referred to as SAGE tags, which are unique to each transcript and which provide a number of advantages for transcriptome analyses in many organisms. SAGE has been employed to investigate gene expression during the early stages of root nodulation, and 407 and 428 tag species have been identified as significantly upregulated and downregulated during the early stage of nodulation, respectively (Asamizu et al. 2005). Detailed information on SAGE analysis is available through a web database at *http://est.kazusa.or.jp/en/plant/lotus/EST/index.html*.

DNA arrays for transcriptome analysis of *L. japonicus* have been produced using PCR-amplified cDNA from several sources, including 4,048 clones from flower buds (Endo et al. 2002), 5,376 partially redundant clones from root nodules (Colebatch et al. 2004), and most recently, 18,144 non-redundant clones derived from a variety of organs (Kouchi et al. 2004). Biological processes that have been studied with these arrays include anther development (Endo et al. 2002), and the development and differentiation of root nodules (Endo et al. 2002; Kouchi et al. 2004). More than 1,000 plant genes that are induced during nodule development and differentiation have now been identified. In addition to the cDNA arrays, oligonucleotide arrays have been developed based on EST, cDNA, and genomic sequence information to represent all known and predicted genes of *L. japonicus* and *Mesorhizobium loti*. A transcriptome atlas of *L. japonicus* has been constructed using these arrays, which should be valuable for studying various processes of fundamental and agricultural importance.

8.5.5 Proteomics

Proteomics is the study of the protein complement of an organism and is facilitated by high-throughput mass-spectrometry, which provides information on the exact mass and/or sequence of proteins and protein-derived peptides. This information, together with genome sequence data and inferred protein sequence data, can be used to identify proteins and to follow changes in protein expression through time and space in an organism. Recently, *Lotus* has been the subject of several proteomic studies; for instance, nanoscale liquid chromatography, which separates proteins or fragments of proteins, obtained from enzymatic digestion, was coupled with tandem mass spectrometry to identify proteins associated with the symbiosome membrane that surrounds Rhizobia in infected nodule cells (Wienkoop and Saalbach 2003). The proteomic analysis of seed development has also been reported (Dam et al. 2009), and detailed information on the analysis data can be investigated further using the Web interface found at *http://www.cbs.dtu.dk/cgi-bin/lotus/db.cgi*. This site has a number of options for viewing and cross-referencing.

8.6 Scope for Future Applications

8.6.1 Metabolites Survey in Lotus Species

Secondary metabolites are one of the most important components in plants considered for industrial use.

In legumes, several specific metabolites have been identified and applied to therapeutic and industrial use. With the aim of investigating potential applications for *Lotus* species, as well as understanding the biochemical processes involved, large-scale metabolomic analyses have been carried out in *L. japonicus*.

Gas chromatography coupled to mass-spectrometry (GC-MS) is currently the major platform for metabolomics in *L. japonicus* (Colebatch et al. 2004; Desbrosses et al. 2005). To facilitate metabolite identification from GC-MS data, a reference library of mass-spectral metabolite tags (MSTs) has been produced for *L. japonicus* and other species (Wagner et al. 2003; Colebatch et al. 2004) and can be found at *http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html*. Using the MST database and software for chromatogram peak area integration, it is possible to quantify and compare metabolite levels in different biological samples. Metabolomics data obtained in this way have been combined with parallel transcriptomics data to yield further insight into the metabolic differentiation that occurs during nodule development (Colebatch et al. 2004).

Metabolomic approaches have also been used to investigate the biosynthetic pathways for nitrile glucosides, the two cyanogenic glucosides, linamarin and lotaustralin, and terpenoides in *Lotus* (Forslund et al. 2004; Arimura et al. 2005). These compounds are thought to play a role in plant–herbivore or plant–insect interactions. The integration of "omics" data from different levels, such as metabolomics with transcriptomics (Colebatch et al. 2004), is beginning to have a significant impact on our understanding of biochemical processes in *L. japonicus*.

Along with the efforts on metabolite analysis, investigations of the genes responsible for the synthesis of legume-specific secondary metabolites are underway. One such approach has involved the extensive analysis of the genes encoding the enzymes involved in the biosynthesis of the legume-specific 5-deoxyisoflavonoid of *L. japonicus* (Shimada et al. 2004, 2007). The findings from detailed analyses of the structures and functions of paralogous genes in the *L. japonicus* genome suggest the functional diversification of the multigene families, due to gene duplication, followed by the accumulation of nucleotide substitutions. The genome-wide analyses of gene families involved in the biosynthesis of legume-specific metabolites is providing insights into the molecular evolution of legume-specific biosynthetic genes, as well as suggesting possible applications of these genes for genetic engineering.

8.6.2 *Application of* Lotus *Species in Bioremediation*

The possibility of the use of *Lotus* species for the purpose of bioremediation has been considered as another potential application.

In polluted soils, the presence of toxic inorganic compounds, such as heavy metals, has an important impact on the resident microflora, which shows significantly less variation in polluted areas. With respect to *Lotus* species, some survive in rather extreme conditions such as those existing in contaminated soils. Taking into account that symbiotic interactions between species of the genus *Lotus* and *Rhizobium* strains can be effective, ineffective or parasitic, according to the particular combination, nodulation tests were evaluated with different *Lotus* species. Several parameters were also analyzed such as population size, nitrogen fixation capacity, genetic diversity, and mercury and arsenic tolerance. The results suggested that certain symbiotic relationships between *Lotus* and *Rhizobium* symbiosis seem to be particularly well adapted to adverse environmental conditions and might be an appropriate tool for the bioremediation of polluted soils (Castro et al. 2007).

8.7 Gene Flow from Transgenic *Lotus* Species

Currently, no cultivar of *Lotus* species has been released through genetic transformation. However, risk assessment for transgenic *Lotus* species has been conducted in advance by measuring pollen dispersal levels. The survey was carried out using transformed plants of *L. corniculatus* as the pollen donors and non-transgenic plants as the recipients (De Marchis et al. 2003). As a result, transgene flow to non-transgenic plants was detected up to 18 m from a 1.8 m^2 donor plot in one location. In a second location, pollen dispersal was detected up to 120 m from a 14 m^2 donor

plot. Therefore, transgene flow does occur in *Lotus* species, and the size of the transgene source plot appears to influence the range of gene flow. This report will be the basis for suitable countermeasures on gene flow control in *Lotus* species.

8.8 Future Outlook

The accumulating nucleotide sequences and their positional information in the genome of *L. japonicus,* as well as *Medicago truncatula* and soybean, have revealed characteristic features of the legume genomes. A rapidly advancing characterization of these three legume genomes will undoubtedly enhance ongoing comparative genomic analyses (Choi et al. 2004, 2006; Zhu et al. 2005; Cannon et al. 2006). As the molecular characterization of induced variation, usually connected with single-locus traits, continues to advance, a shift towards legume-specific multigenic traits or QTLs can easily be envisaged. The single-locus approach will validate individual players in key developmental processes, while the progression of QTL cloning will define gene networks in a more holistic context involving the interplay between intrinsic and exogenous factors that determine plant productivity.

The transfer of knowledge acquired from model plants, mainly through orthologous gene sequences and DNA markers, results in identification and isolation of the corresponding genes using either genomic and/or cDNA libraries and common DNA markers on genetic linkage maps in crop plants. The creation of a larger number of "common words" will be crucial for the more efficient transfer of knowledge and for facilitating the exchange of knowledge between researchers working on legumes. The LOTASSA (LOTus Adaptation and Sustainability in South-America) program is a tangible example of the current efforts towards the practical application of genome information in model legumes. LOTASSA is the acronym for a project entitled "Bridging Genomics and Agrosystem Management: Resources for Adaptation and Sustainable Production of forage *Lotus* species in Environmentally Constrained South-American Soils," which has been recently selected for funding by the European Union (EU) International Cooperation (INCO) Program. The global objective of LOTASSA is to develop superior biological and genetic resources to (1) assist and speed up selection of *Lotus* genotypes that are more tolerant to abiotic stresses, and (2) improve the productivity, sustainability, and quality of *Lotus* pastures in environmentally constrained areas of South America. For this objective, LOTASSA will exploit the close genetic relationship between the model plant *L. japonicus* and cultivated *Lotus* species. In this respect, LOTASSA represents a pioneering project where basic and applied plant researches are combined for a common goal. This international experience provides a great advancement in the genetics, physiology, and microbiology of *Lotus* towards more productive and sustainable pastures for fattening animal livestock in the Southern Cone region of South America.

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Chapter 9
Lupinus

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9.1 Basic Information on *Lupinus* Genus

9.1.1 Distribution and Centers of Diversity

Lupinus is a relatively large genus and one of the most geographically widespread with a rich diversity of species that are divided into two major groups – the Mediterranean/North and East African "Old World" species and the North and South American "New World" group comprising the greater number of species. Parallel indigenous domesticates occur in the Mediterranean and the Andes regions; however, when compared to nearly all other food crops, lupins have only recently been the focus of modern crop breeding. Wild and landrace lupins are distributed across climatic ranges from subarctic regions (Alaska), Mediterranean, and semi-desert climates; highland and mountain regions of East Africa, Mexico, the Andes, and High Rockies; the warm temperate climates of southeastern USA and subtropical regions of eastern South America (Gladstones 1998). They include simple and compound leaved, herbaceous annual and herbaceous to shrubby perennial plant types. The Old World group has only annual species. Generally, lupins are plants of open and well-lit habitats, which do not tolerate shading (Gladstones 1998), and prefer well-drained soils with from acid to neutral pH with limited distribution into the alkaline range – the rough-seeded species showing some tolerance to above neutral pH. A recent exception regarding soil pH preference is the newly described 13th Old World species, *L. mariae-josephi* H. Pascual, which is reported to prefer calcareous soils (Pascual 2004; Pascual et al. 2006).

Based on studies that assessed genetic similarities, the centers of diversity of *Lupinus* are: (1) North and Central America and Andean South America, (2) Atlantic South America, and (3) the Mediterranean and northern and eastern African region (Hondelmann 1984; Planchuelo 1994; Ainouche and Bayer 1999; Maciel and Schifino-Wittmann 2002). The distribution maps of the Mediterranean (Old World) species can be found in Gladstones (1974, 1998). Within the major Old World species groupings, there are more local regions considered to be the species centers of diversity. For *Lupinus albus* L., the semi-domesticated large-seeded forms (var. *albus*) have long been cultivated around the Mediterranean and in the Nile valley, but the primary center of diversity is implied from the reported distribution of the wild *L. albus* var. *graecus*, which includes the southern Balkans (Greece including Crete, Albania, former Yugoslavia) and possibly into northeastern Greece, southern Italy, and western Turkey (Perrino et al. 1984; Clements and Cowling 1990; Gladstones 1998). The natural distribution of the derived cultivated landraces, *L. albus* var. *albus* is circum northern Mediterranean basin, the mid-Atlantic islands (Azores), the Canary Islands, North Africa (Morocco, Algeria, Tunisia) and the Nile valley, Kenya, and Ethiopia (Buirchell 1992; Gladstones 1984, 1998; Neves-Martins 1994). Generally, *L. albus* is distributed on mildly acidic or neutral soils of light to medium texture. Some occurrences on alkaline soils are reported (Christiansen et al. 1999, 2000).

Lupinus luteus L. is more confined to sandy, acidic soils and natural populations are much less widespread than *L. albus* or *L. angustifolius* L. and truly wild

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forms are rare. The proposed place of origin is the Iberian Peninsula (Gladstones 1974), and the primary current distribution center is in the western parts of this region. Smaller-seeded, likely wild types occur in the high rainfall areas of Portugal and Northwest Spain, with some reports of occurrences in southern Spain, southern Italy (Calabria, Sicily), Greece, Turkey, coastal Morocco, and Israel and Lebanon (Gladstones 1974; Cowling 2001; Kurlovich 2002b). Occurrences in the eastern Mediterranean support the hypothesis of a temperate western Asian origin of the smooth-seeded lupin species (Gladstones 1998). *L. luteus* landraces are scattered along with the wild forms and as escapes from cultivation or used as a garden ornamental. *Lupinus hispanicus* Boiss. and Reut., which is closely related to *L. luteus* (included in the *Lutei* section described by Nowacki and Prus-Glowacki 1971) but whose distribution extends to higher altitudes, is found mainly in Spain and Portugal (Gladstones 1974). *L. hispanicus* ssp. *hispanicus* is found at moderate altitudes in southern and central Spain and *L. hispanicus* ssp. *bicolor* at higher altitudes up to 1,500 m in northwestern Spain and northern Portugal, sometimes on poorly drained soils. There have been some reports of occurrences in Turkey and northern Greece (Gladstones 1998; Clements and Cowling 1990; Cowling 2001). *L. hispanicus* is generally distributed on sands to sandy loams, which are acidic to very acidic (Gladstones 1974). *Lupinus micranthus* Douglas is relatively rare but is the second most widespread Old World species after *L. angustifolius* (Gladstones 1974). Its distribution is around the perimeter of the Mediterranean basin and this implies its translocation through human activity over time. It is found not only on mildly acidic to alkaline soils, frequently on sandy loams, but also on coarse sands and heavier and more calcareous soils.

The Aegean region is the possible center of diversity of *L. angustifolius*, with small-seeded, finer-leafed genotypes occurring particularly at higher elevations in northern Greece and some islands (Clements and Cowling 1994; Cowling 2001). Gladstones (1998) noted the existence of possible wild types distributed in North Africa and Iberia. A continuous range from small-seeded to larger-seeded types is found, larger seeds consistent with selection activity by early farmers. *L. angustifolius* natural populations are more widespread than the other Old World *Lupinus* species. Generally, *L. angustifolius* is found on acid, well-drained, non-calcareous, light to medium textured soils.

The seven rough-seeded *Lupinus* species (which includes the possibly extinct *L. somaliensis* Baker) mostly have a very restricted natural distribution, and their habitats generally show little overlap. These species still exist as wild plants with evidence of some human selection for larger seeded or ornamental types in *L. pilosus* Murr., *L. digitatus* Forsk, *L. palaestinus* Boiss., and *L. atlanticus* Gladst. (Gladstones 1974, 1998). Their habitats range from desert valleys to tropical highlands; from high mountain regions to coastal plains; from acidic soils to highly calcareous soils. They are distributed in a wide range of environments and soil types, although predominantly from neutral to alkaline soils. Some limited overlap occurs between *L. pilosus* and *L. palaestinus* in Israel. Hybrids between *L. pilosus* and *L. palaestinus* may occur where the two species grow together (Clements et al. 1996). Little penetration of the rough-seeded lupins into the northern Mediterranean has occurred with their specialized adaptation to semi-desert and warm Mediterranean conditions. *L. pilosus* has a diverse distribution from the mountainous regions of Crete and Turkey to the Greek Islands to the coastal areas of Israel and Syria. It grows on a wide range of soil types having been collected from coastal sandy soils to loamy clays with limestone present. It generally occurs on sandy soils of neutral to alkaline pH. The species seems well adapted to a number of climatic environments, and there is considerable variation within this species. *L. palaestinus* is a low-growing plant with much basal branching and has long inflorescences. Its natural range extends from the central and southern plains of Sharon and Philistia, down the coast of Israel, and on the Sinai Peninsula in the Jebel El Tih. *L. atlanticus* Gladst. is a distinctive species restricted to the Atlas and Anti Atlas mountains of Morocco at altitudes less than 1,700 m but greater than 460 m. The northern extent of its habitat is around Beni Mellal, and it is found on the western slopes of the Atlas Mountains. In the anti-Atlas Mountains, it is found in the Valleys of the central mountains around Tanalt, in the valleys around Tafraoute and on the Kerdous Plateau. The southern boundary is unknown but is restricted to above 250 mm rainfall.

Lupinus princei Harms isolated to the highlands of Kenya, Tanzania, and southern Ethiopia at elevations

between 1,700 and 3,000 m. *L. digitatus* is known from a number of regions in southern Sahara, mostly associated with mountainous areas. It has been found on the border of Chad and Libya, Niger and Algeria, in the Senegal Valley, the northwest Sahara, and in the Nile Valley. Limited collections occur in international seed banks, the most known samples coming from Southeast Egypt. The species is endangered due to desertification within most of its range. The extent and status of the distribution of *L. somaliensis* is unknown but may extend from the region of its type specimen in the Golis Range of northern Somalia into Ethiopia. No accessions are known to exist in genebanks.

The New World lupins are distributed across a wide range of climates including alpine, temperate, and subtropical. The centers of diversity are considered to be within these regions and are primarily North and Central America, the Andean South American region, and the Atlantic South American region, which encompasses the majority of the primitive simple-leafed species (Fig. 9.1). Planchuelo (1994) divides the North and Central American region into the southeastern subregion (SEN) and Mountain range from Alaska to Central America subregion (MAC). The SEN group consists of four simple-leafed species (thought to originate from Brazil) in coastal North Carolina to the Mississippi in the Gulf of Mexico. The MAC group of compound-leafed lupins extends from the Aleutian Island in Alaska, along and on both sides of the mountains and ranges in North America to the Sierra Madre in Mexico and Central America. The group includes ornamental and forage species *L. polyphyllus* Lindl., soil conservation species *L. arboreus* Sims and *L. nootkatensis* Donn ex Sims, and the ornamental *L. mexicanus* Cerv. ex Lag. (synonyms *L. hartwegii*, *L. ehrenbergii*). The South American region is divided into the Atlantic subregion (ATL) and Andean subregion (AND). The ATL group consists of a large number of perennial simple and compound-leafed species and a small group of variable annual species. It includes the *L. gibertianus* C.P. Sm.-*L. linearis* Desr. complex. The group is distributed across eastern Brazil, Uruguay, Paraguay, and central and eastern Argentina. The AND group covers the geographic region from the mountain slopes on both sides of the Andes, from Venezuela through Colombia, Ecuador, Peru, Bolivia, Chile, and Northwest Argentina to the plains of Patagonia in the

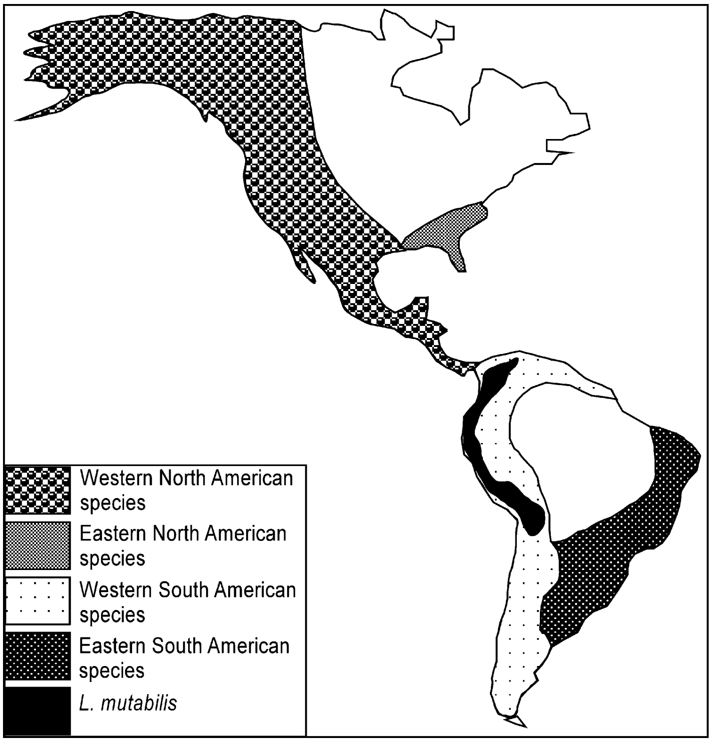


Fig. 9.1 Approximate distribution regions of New World lupin species including *L. mutabilis* [from Planchuelo-Ravelo (1984), Mujica (1994), Planchuelo (1994), and various collection site data sources]

south. The species consist of perennial and annual compound-leafed species. The range in topography and microclimates create a large ecological diversity of plant types (Planchuelo 1994). Within the AND group is *L. mutabilis* Sweet, the only crop species from the region. Domesticated independently from Old World crop lupins in the Mediterranean, *L. mutabilis* populations exist in semi-domesticated form, with non-shattering pods, large, permeable seed, reduced seed coat pigmentation and a more or less annual life cycle. Reports of its cultivation are from as far north as Venezuela through Colombia, Ecuador, Peru, Bolivia, to Chile and northern Argentina (Williams 1979; Blanco 1982; Planchuelo and Dunn 1984; Mujica 1994; Planchuelo 1994; Fig. 9.1). Research by Eastwood and Hughes 2008 had failed to reveal any occurrences of *L. mutabilis* in anything resembling natural vegetation, suggesting that the species may only be found in cultivation with occasional individuals persisting in fallow fields and their margins after the previous crop. Based on field, herbarium, and DNA sequence data, they suggest *L. piurensis* C.P.Sm., a species with similar morphology but differences particularly with fruits and seed as the most likely wild progenitor of domesticated *L. mutabilis*. It is distributed on the western slopes of the Andes between 1,650 and 3,300 m in northern Peru (Eastwood and Hughes 2008).

9.1.2 *Taxonomy and Phylogeny of the Genus*

Historically, the Leguminosae family has been divided into three subfamilies: Caesalpinieae, Mimosoideae, and Papilionoideae, the latter subfamily forming the grain legumes. This subfamily is monophyletic, and recent studies agree on a series of clades within it that are well supported and have received informal names. Of the seven clades, there are four within the Papilionoideae that include most of the economically important food and feed legumes. Of these, the genistoids include the *Lupinus* genus (Gepts et al. 2005; Cronk et al. 2006). Despite the broad distribution and relatively variable morphology within *Lupinus*, its unity is unquestioned, and the majority of authors agree in assigning the genus to the tribe Genisteae. Some variations to this taxonomic citation, however, do occur. A more recent study (Ainouche and Bayer 1999), based on internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA using 44 *Lupinus* taxa and five outgroup taxa, clearly supported previous classifications that the *Lupinus* genus is a strongly monophyletic genus and should be included in tribe Genisteae but as a distinct lineage from the Genistinae ("Cytisus-Genista complex") as subtribe Lupininae (Hutch.) Bisby. The results were consistent with serological data (Cristofolini 1989) and molecular-based phylogenies of the Papilionoideae (Doyle et al. 1997; Käss and Wink 1997).

There is a wide phylogenetic distance between lupin and the cool season food legumes that include pea, lentil, faba bean, and chickpea (Dwivedi et al. 2006). The lupin genome is distinct from these other species in that its chromosome number varies widely (from $n = 12$ to $n = 26$), it has a significant number of duplicate loci for relatively conserved isozymes (Wolko and Weeden 1990a), and it has a smaller DNA content per haploid complement (Weeden et al. 2000). Compared to other crop species, cytological work in *Lupinus* is relatively deficient, and this may be partly attributed to the large number of chromosomes (range from $2n = 32$ to 52), similarity in chromosome size and shape such that pairs are not easily defined, and difficulty in distinguishing individual species through chromosome length or arm ratios.

The detailed taxonomy of lupins has been dealt with by several authors, including Gladstones (1974, 1984, 1998), Plitmann and Heyn (1984), and Planchuelo (1994). Until the major taxonomic revision of Gladstones (1974), the taxonomy of the Old World lupins was confused as a result of mistakes from the early years of Linnaean taxonomy. The taxonomy of the New World lupins has proven difficult due to the large number of apparent species and intermediates resulting from outcrossing and a high degree of phenotypic plasticity across their habitats (Planchuelo 1994).

Thus, the two geographically separate groups – the New World species and the Old World lupins – together occupy habitats from sea level to alpine tundra up to 4,000 m altitude. Species vary from annual to perennial with growth habits from acaulescent or small prostrate to tree-like shrubs reported to be of 4.5 m (Dunn 1984) or even to 8 m high with woody trunks of 30 cm across such as in *L. jaimehintoniana*

B.L. in Mexico (Turner 1995). The main features that distinguish the genus *Lupinus* are large and numerous flowers on terminal racemes; flowers with a deeply cleft calyx, erect standard petal; wings connate at the apex; keel incurved, beaked, and enclosed within the wings; stamens 10, alternately long and basifixed, short, and versatile; ovary sessile; style incurved, glabrous; stigma terminal; pod oblong, more or less compressed, septate between the seeds, valves thick and leathery, dehiscent; cotyledons thick and fleshy (Allen and Allen 1981). The distinctiveness of the New World and the Old World lupins has been supported by various authors who based upon the analyses of seed globulin patterns (Przybylska and Zimniak-Przybylska 1995; Przybylska and Przybylska 1997), protein serology (Cristofolini 1989) and general taxonomic reviews (Plitmann and Heyn 1984).

In addition to the geographic division of the Old and New World, lupins can be separated on leaf characters, one with simple leaves of approximately 26 species and the other with typically palmate compound leaves (Planchuelo 1994). The other subgeneric division is made on seed testa structure (Plitmann and Heyn 1984) – seven species, which have rough testa (section Scabrispermae), are all distributed in the Old World, while the rest are typically smooth (section Malacospermae) and include the economically important species, *L. angustifolius*, *L. luteus*, *L. albus*, and *L. mutabilis*. The rough-seeded micromorphology is characterized by the pluricellular tubercules of the seed coat, which are not present in the smooth-seeded species (Lush and Evans 1980; Plitmann and Heyn 1984; Ainouche and Bayer 2000). Support for the separation of the species into the groupings rough and smooth-seeded is provided by studies using flavonoids (Williams et al. 1983), isozymes (Wolko and Weeden 1990b), seed globulin patterns (Przybylska and Zimniak-Przybylska 1995; Przybylska and Przybylska 1997), cytology (Plitmann and Pazy 1984), coat structural comparisons, and DNA (Käss and Wink 1997; Ainouche and Bayer 1999) (Fig. 9.2).

9.1.3 Old World Lupin Species

The generally accepted taxonomy of the Old World lupin species is that of J. S. Gladstones whose comprehensive studies at several herbariums from 1968 resulted in the publication of revised taxonomy, history, and use of lupins (Gladstones 1970, 1974). This classification is supported by other studies (Salmanowicz and Przybylska 1994; Ainouche and Bayer 1999; Naganowska et al. 2003b) and is highly congruent with recent ITS-DNA data (Ainouche and Bayer 1999). Nowacki and Prus-Glowacki (1971) distinguished five groups of species on serological grounds, and these agreed well with morphology and

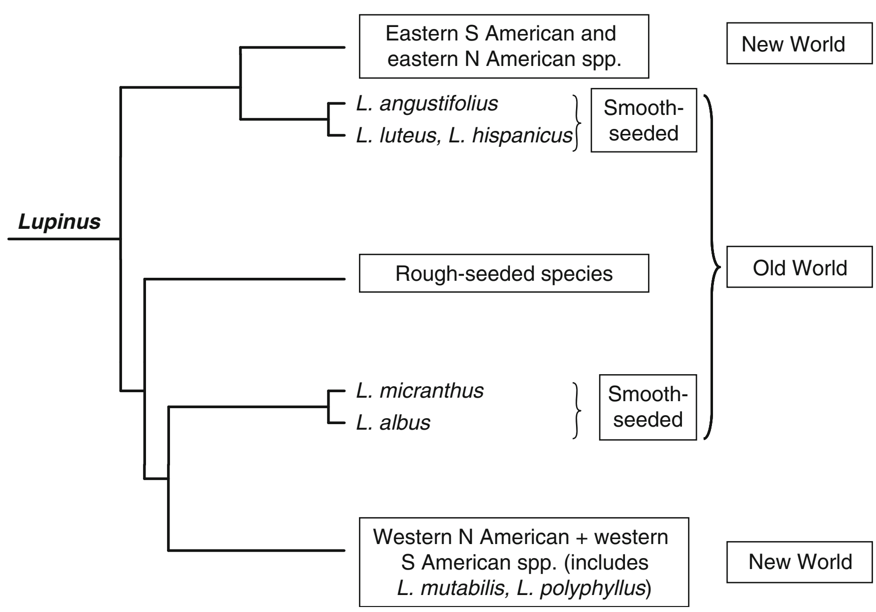


Fig. 9.2 Generalized diagram showing phylogenetic relationships among Old and New World *Lupinus* [based on information from Käss and Wink (1997), Ainouche and Bayer (1999), Merino et al. (2000), Hughes and Eastwood (2006), Eastwood and Hughes (2008), and Eastwood et al. (2008)]

crossability among the Old World group as discussed by Gladstones (1984). Despite showing a range of chromosome numbers, the morphological, serological, genetic, isozyme, and interspecific crossing ability evidences show that the rough-seeded species from the Old World are very homogeneous (Gladstones 1974; Plitmann and Heyn 1984; Cristofolini 1989; Wolko and Weeden 1990a; Gupta et al. 1996) and the most strongly supported clade in the Old World group (Ainouche and Bayer 1999). Carstairs et al. (1992) placed the rough-seeded lupins, based on cytogenetic and crossability studies into three groups: the Princei group (*L. princei*) in equatorial Africa, the Atlanticus group (*L. atlanticus*, *L. cosentinii* Guss. and *L. digitatus*) in northwestern Africa, and the Pilosus group (*L. pilosus* and *L. palaestinus*) in the eastern Mediterranean. *Lupinus anatolicus* Święc. was proposed after a smooth-seeded seed sample was collected from the hills near Efez, Turkey (Święcicki 1988; Ainouche and Bayer 2000); however, accessions of *L. pilosus* containing smooth seeds have been found in Syria (Buirchell 1999), and based on the other evidence, *L. anatolicus* may be an ecotype or a natural mutant of *L. pilosus*.

L. palaestinus has the same chromosome number as *L. pilosus*, and their ability to experimentally intercross, albeit with considerable genetic barriers, suggests a close relationship between them. DNA contents were very similar for *L. pilosus* and *L. palaestinus* compared to *L. cosentinii* and *L. atlanticus* (Ghrabi et al. 1999). Other studies confirming the *L. pilosus*/*L. palaestinus* relationship are found for ITS data (Ainouche and Bayer 1999), seed proteins (Salmanowicz 1999), and interspecific crossing ability (Carstairs et al. 1992), but none have suggested combining them as one species. *L. cosentinii* ($2n = 32$) has been referred to in the past as *L. cosentini*, *L. varius*, and *L. digitatus*, but clarification was established both by Gladstones (1970, 1974) and through chromosome counts. Moroccan or Tunisian genotypes differ with respect to some morphological characters from those in the northern shores of the Mediterranean and the Australian naturalized populations. Based on ITS data, *L. cosentinii* is separated from the *L. pilosus*/*L. palaestinus* and the *L. digitatus*/*L. atlanticus* subdivisions but is more closely related to the latter two (Ainouche and Bayer 1999). This closer relationship is reflected in seed protein studies (Salmanowicz and Przybylska 1994; Salmanowicz 1999).

L. digitatus ($2n = 36$; Plitmann and Heyn 1984) has been referred to as *L. tassilicus* Maire, but morphological and chromosome evidence support the synonymy of *L. tassilicus* and *L. digitatus* (Gladstones 1974; Carstairs et al. 1992). Synonyms for *L. atlanticus* were similarly clarified by Gladstones (1974). *L. atlanticus* has the same chromosome number as *L. princei* ($2n = 38$; Carstairs et al. 1992), but crossing and cytological studies suggest closest genetic affinity to *L. cosentinii* (Roy and Gladstones 1988; Carstairs et al. 1992; Gupta et al. 1996) with some affinity to *L. pilosus* (Gladstones 1998). The geographically isolated *L. princei* is relatively distinct morphologically but is identified in phylogenetic studies to clearly belong to the rough-seeded group (Käss and Wink 1997). Naganowska et al. (2003b) found it to have a nuclear DNA content closer to the smooth-seeded species (very similar to *L. micranthus*), and Carstairs et al. (1992) noted that it had the longest chromosomes among the rough-seeded lupins analyzed. It has failed to produce viable seeds when crossed with other rough-seeded species (Carstairs et al. 1992; Gupta et al. 1996), and this along with its morphological distinctiveness supports its status as a separate species. Further taxonomic evaluation and exploration appears worthwhile for the rough-seeded lupins because of the smaller numbers of available accessions, the tendency for ecogeographical isolation among many of these species, and the existence of numerous isolated populations in their habitats in North Africa and the eastern Mediterranean.

The smooth-seeded Mediterranean group is a much less homogeneous, more widely separated group, and has major genetic barriers between most species (Plitmann and Heyn 1984). Genetic, biochemical, and serological studies support this separation (Cristofolini 1989; Wolko and Weeden 1990b). The smooth-seeded Old World lupins were separated into four distinct sections (*albus*, *angustifolius*, *luteus*, *micranthus*), all monospecific apart from the *L. luteus*–*L. hispanicus* complex (Nowacki and Prus-Glowacki 1971; Gladstones 1984). ITS genetic evidence resolves them into two distinct clades, and within these clades, all species are well-defined (Ainouche and Bayer 1999). One of the clades defined by Ainouche and Bayer (1999) consisted of three species, *L. angustifolius* ($2n = 40$), *L. luteus*, and *L. hispanicus* (both $2n = 52$). This indicated a close relationship between the Angustifoli and Lutei sections described by

Nowacki and Prus-Glowacki (1971) and Gladstones (1984), despite them being relatively distinct plants in morphology and cytology (Fig. 9.2). *L. luteus* was proposed as being closer to *L. micranthus* based on some similar morphological traits and because both have the same chromosome number (Gladstones 1984). Talhinas et al. (2003) also showed the phylogenetic connection between *L. angustifolius* and *L. luteus*–*L. hispanicus* using several molecular marker types. Some support for the connection between *L. luteus* and *L. angustifolius* might also be provided by the existence of a "foveolate" seed coat pattern found in *L. angustifolius* samples from North Africa that were similar to the appearance of *L. luteus* seed coat types (Ainouche and Bayer 1999).

L. luteus and *L. hispanicus* (both $2n = 52$) were shown by Ainouche and Bayer (1999) to have genetic affinity but were clearly distinct based on nucleotide changes. Both species are distributed in the Iberian peninsular and separated by reproductive barriers; however, Święcicki et al. (1999) produced interspecific progeny from *L. luteus* × *L. hispanicus* subsp. *hispanicus*. Israeli forms of *L. luteus* have been referred to as subspecies *orientalis* and were typified by smaller seeds and were later flowering than the western Mediterranean types. Gladstones (1998), from observations of the different forms growing together, did not support their classification as a subspecies. *L. hispanicus* Boiss. and Reuter has been divided into subspecies by Gladstones (1974) who describes two fairly distinct forms existing in the Iberian Peninsula. Subspecies *hispanicus* was in accordance with the original type of Boissier and Reuter, which is similar to *L. luteus*, but flowers and seeds differentiate it reasonably clearly. *L. hispanicus* ssp. *hispanicus* and ssp. *bicolor* did not form fertile hybrids with *L. luteus*, but within and between *L. hispanicus*, there was a relatively high production of fertile F_1 progeny with intermediate characteristics (Gladstones 1984).

The albus section comprises *L. albus* ($2n = 50$) with its subgroups var. *albus* and wild forms, var *graecus*, and the previously described species *L. graecus*, *L. juvoslavicus*, and *L. vavilovi* are considered to be blue-flowered, dark-seeded wild forms synonymous with var. graecus (Gladstones 1974, 1984). A numerical taxonomic study by Nowacki et al. (1988) using 19 morphological characters on 19 genotypes recommended the separation into separate species; however, earlier crossing studies showed no genetic barriers between wild forms and var. *albus*. Recent RAPD-based evidence (Przyborowski and Weeden 2001) also tends to support the proposal of botanical varieties rather than separate species. A strict consensus tree clade described by Ainouche and Bayer (1999) grouped *L. albus* ($2n = 50$) with *L. micranthus* ($2n = 52$), and together with the isozyme data (Wolko and Weeden 1990b) suggests the close affinity of *L. micranthus* with the smooth-seeded Old World species. Clements et al. (1993) noted that *L. albus* and *L. micranthus* were similar among the Old World species in their prolific production of proteoid (cluster) root structures. Other evidence places *L. micranthus* somewhere between the smooth-seeded and rough-seeded Old World lupins (Williams et al. 1983; Cristofolini 1989; Wolko and Weeden 1990a). Further, morphological parameters of fruits and seeds together with peroxidase enzyme data suggested that *L. micranthus* was closer to *L. angustifolius* than to *L. albus* var *graecus* (Drossos et al. 1996). The evidence, however, generally supports that *L. micranthus* and *L. albus* are genetically closer to the rough-seeded group than are *L. luteus*, *L. hispanicus*, and *L. angustifolius*. Additionally, *L. albus* (and therefore *L. micranthus*) appears to have an intermediate position between the Old World and western New World species (Fig. 9.2; Käss and Wink 1997; Ainouche and Bayer 1999).

L. angustifolius has had a range of synonyms associated with it in the past, including *L. varius* L., *L. linifolius* Roth., *L. reticulatus* Desvaux, and *L. philistaeus* Boiss., all of which referred to the shorter, finer-leafed, and smaller-seeded wild types. These were in contrast to the larger-leafed, larger-seeded types referred to as *L. angustifolius*, which probably resulted from some deliberate selection over time. *L. opsianthus* Atab. et Maiss. referred to a small-seeded wild ecotype from Portugal and has been noted to have thin seed coats. Some past subdivisions of the species include *L. angustifolius* var. *basalticus*, a form with dark flowers, broad leaves, and pods, distributed in Israel on basalt soils that contrasted to "typical" var. *L. angustifolius* growing on coastal sandy soils. Gladstones (1984) assessed these types to be very similar in morphology to cultivars of northeastern Europe that may have been introduced to Israel. Little or no genetic incompatibility is reported between the wild and primitive genotypes of *L. angustifolius*. Some minor fertility reduction in some crosses between modern cultivars and wild types of eastern Mediterranean

origin were noted by Gladstones (1984). Given the large spectrum of variation across the naturally occurring populations of *L. angustifolius*, the division between typical wild versus larger-seeded types is difficult. Recently, Kurlovich and Stankevich (2002) put forward a number of subdivisions especially for *L. angustifolius*, *L. albus*, and *L. luteus*, including subspecific and botanical variety groupings. Further, a large number of agroecotypes were described (Kurlovich 2002b). Although of questionable taxonomic status, such subgroupings could be useful for the development of core collections by gene banks and selection of representative types to include in breeders' crossing programs aimed at broadening the genetic base.

9.1.4 The New World Lupin Species

There has been a lack of monographic synthesis for the New World lupin species. A review of the South American lupins was the Species Lupinorum published by C. P. Smith from 1938 to 1945 and then by Macbride (1943). Several hundred species were described based only on superficial characters, and many of the taxa were considered later to by synonyms (Planchuelo-Ravelo 1984). Maisurjan and Atabiekova (1974) distinguished 12 sections among the New World lupins, and since then, the studies conducted by D. B. Dunn, A. M. Planchuelo and others (Planchuelo-Ravelo 1984, 1991; Planchuelo 1994, 1999, 2000), and C. Hughes (Hughes and Eastwood 2006; Eastwood et al. 2008) using classical and molecular taxonomy have gone some way to clarify the group. The latest estimates of *Lupinus* are that the genus comprises a total of approximately 280 species including approximately 85 and 100 species in the Andean and western North American groups, respectively (Eastwood et al. 2008). Dunn (1984) demonstrated the heterogeneity of species populations, noting that the *L. mexicanus*–*L. exaltatus* Zucc. complex in Mexico contains both annual and perennial species, which are morphologically indistinguishable and interfertile. Planchuelo (1978) reviewed taxa from Argentina and reduced from 85 to 29 the number of species for that region. Further taxonomic studies of the species of that region such as the *L. gibertianus*–*L. linearis* complex, are ongoing. Many of the North American and Mexican species have been reviewed to reduce the taxa from 200 to 21 species. Taxonomic research and chromosome counts for Mexican and Central American lupins are still required to review the large number of published names for the region (Planchuelo 1999; Eastwood and Hughes 2008).

The predominant chromosome number for the New World lupins is $2n = 48$. Some species or individuals for which counts are recorded have $2n = 96$, and hybrids among Alaskan species with $2n = 48$ and 96 have been noted. Turner (1994) reported $2n = 36$ for the majority of closely related species in northeastern Mexico with an exception of $2n = 24$ for *L. caballoanus* B.L. Turner, although recent investigations have shown that species to have $2n = 48$ (Conterato and Schifino-Wittmann 2006). Recent studies confirmed the existence of three species in South America with $2n = 36$ chromosomes, *L. albescens* Hook. Et Arn., *L. paraguariensis* Chodat et Hassl., and *L. multiflorus* Desr. (Perrisse et al. 2000). Dunn (1984) also refers to a count of $2n = 24$ in a specimen of *L. aridus* Dougl., although this contrasts to $2n = 48$ reported elsewhere (Conterato and Schifino-Wittmann 2006). A range of southeastern South American species has predominantly $2n = 36$ with exceptions of $2n = 32$ and 34 (Maciel and Schifino-Wittmann 2002). Several studies lend support to the genetic separation of the western New World (western North and South America), the eastern New World (including east-central parts of South America and southeastern USA, Florida), and the Old World species (Käss and Wink 1997; Ainouche and Bayer 1999; Maciel and Schifino-Wittmann 2002). Phylogenetic evidence suggests that the western New World species are possibly closer to the Old World species than they are to the eastern New World species (Ainouche and Bayer 1999; Eastwood and Hughes 2008). The studies also indicate a relatedness (although wide) of the two species *L. albus* and *L. micranthus* to the western New World species (Käss and Wink 1997; Ainouche and Bayer 1999). The Central American species such as *L. mexicanus* and *L. elegans* Kunth (Mexican), and South American *L. mutabilis* (Andean) and *L. microcarpus* Sims (N. and S. America), are grouped together with the western North American species (Ainouche and Bayer 1999). The close relationship between *L. mutabilis* and its possible relatives such as *L. bogotensis* Benth. and *L. cruckshanskii* Hook. (the latter possibly synonymous with *L. mutabilis*), *L. praestabilis* C.P. Sm. and *L. piurensis* to the North American

species is also supported in serological (Cristofolini 1989), isozyme (Wolko and Weeden 1990b), and DNA studies (Käss and Wink 1997; Eastwood and Hughes 2008). Eastwood and Hughes (2008), based on nuclear ITS I and ITS II genes, showed the phylogenetic closeness of two species from Florida, *L. cumulicola* Small and *L. villosus* Willd. (each $2n = 52$), with *L. angustifolius* ($2n = 40$) and *L. luteus*–*L. hispanicus* ($2n = 52$).

9.1.5 Secondary Metabolites and Antinutritional Compounds

Secondary metabolites are active constituents of plants that frequently function in plant regulation, metabolism, and other essential processes and are metabolically expensive for the plant to produce. Some of the compounds have a simultaneous role in the attraction of pollinating or fruit-dispersing animals or as UV protectants (Wink 2006). The major plant secondary metabolites include alkaloids, glycosides, phenolics, uncommon proteins and unusual free amino acids, steroids, essential oils, terpenes, and resins (Jackson 1991). Many of these compounds are antinutritional when present at high concentrations, but some are of interest as pharmacological and nutraceutical substances for animals and humans, an example being the antidiabetic properties of sparteine, lupanine, and 13-hydroxylupanine (Wink 2006). Lupins produce several secondary metabolites that are considered antinutrients; however, unlike soybeans and several other food legumes, they do not need heat treatment to deactivate substances such as the lectins and protease inhibitors found in other genera that reduce protein digestion and availability.

9.1.5.1 Alkaloids

Lupins contain quinolizidine alkaloids, and this group of substances is common in Fabaceae, particularly among the genistoid group of legumes, and is also present in some other unrelated families. Different lupin species have unique alkaloid profiles of usually 4–5 major and several minor alkaloid types. Analysis of these compounds has been used to examine taxonomy among legumes (Käss and Wink 1994, 1995; Wink and Mohamed 2003). Alkaloid level is typically lower in shoot material than in seed. For example, in *L. luteus*, total alkaloid levels range from 1 to 4% in seed and 0.2 to 0.5% in shoot dry matter (Hackbarth and Troll 1956). In the crop lupin species, low alkaloid forms have been developed through the identification of natural and artificial mutant genes, which lower alkaloids in both vegetative and reproductive tissues but retain similar alkaloid ratios to wild types. Low alkaloid breeding lines of *L. mutabilis* can have seed total alkaloid levels as low as 0.001% (average 0.004%) compared to 3.3% for bitter landraces (Clements et al. 2008). Alkaloid levels in lupin crops in Australia are governed by food and feed standards, which specify an upper limit of 0.02% total alkaloids in whole seeds for varieties (Culvenor and Petterson 1986). This has allowed the use of the commercial term Australian sweet lupins for *L. angustifolius* seed exported from Australia, denoting that it is safe both for food and feed purposes.

Lupin alkaloids are toxic to herbivores such as bees, caterpillars, beetles, aphids, locusts, snails, nematodes, rabbits, and cows and have antiviral, antibacterial, and antifungal properties (Wink 2006). The bitter taste reduces palatability (Edwards and Barneveld 1998) and acts as a deterrent to larger mammalian herbivores, which might not initially be affected by pharmacological side effects of eating lupins. The biosynthetic pathway for alkaloids begins with lysine, which is converted to cadaverine that in turn produces the two primary tetracyclic molecules, sparteine and lupanine. Gramine, which is found in some genotypes of *L. luteus* (typical wild types do not have gramine) and in *L. hispanicus*, is an indole alkaloid synthesized from tryptophan and therefore is under different genetic control. Examination of F_2 populations derived from crossing a gramine-containing genotype (cv. Teo) with a non-gramine cultivar (cv. Wodjil) has indicated that the gramine pathway was controlled by a single recessive gene (Sweetingham et al. 2006a). Production of alkaloids is within the chloroplasts in aerial green tissues (Wink 1984). Approximately half of the alkaloids that accumulated in pod and seed tissues of *L. albus* and *L. angustifolius* was due to synthesis in situ and half to translocation principally by phloem (although some in xylem) from other green tissues (Lee et al. 2007). Earlier work had implicated synthesis within all green tissues, including the pods, but with far greater proportions translocated from elsewhere in the plant into seed (Wink 1993). Although further work is needed to look at the in situ synthesis of alkaloids by direct experiments using labeled

precursors and determination of gene expression, it is known that pod wall and developing seed tissues each contain considerable concentrations of chlorophyll during growth and it therefore is expected that production could occur at these sites (Clements et al. 2006).

The major alkaloids found in crop lupin species are: *L. albus*, lupanine, 13-α-hydroxylupanine, sparteine; *L. angustifolius*, lupanine, 13-α-hydroxylupanine, angustifoline; *L. luteus*, gramine (some cultivars), lupanine, cytosine, lupinine; and *L. mutabilis*, lupanine, sparteine, 13-α-hydroxylupanine (Wink et al. 1995; Petterson 1998). New World lupins of northern America have a range of contrasting profiles, some with unique alkaloids (Petterson 1998; Torres et al. 2002). Production of alkaloids as with other secondary metabolites is influenced by interactions between genotype and environment. Potassium deficiency is reported to increase alkaloid levels in low alkaloid (*iucundus* gene) lines but not in high alkaloid *L. angustifolius* genotypes (Gremigni et al. 2001). The point was made that plants grown on K-deficient soils are more susceptible to a range of environmental stresses, which in turn may have had direct effects on alkaloid metabolism. It was also found in that study that alkaloid levels were consistently higher in seed from plants grown in the glasshouse, compared with seeds produced in field experiments. It was suggested that this may have been due to the effects of high light energy and air temperatures in a glasshouse and to low rooting volume. Seeds from sweet lupins grown over summer in Western Australia (normally grown during the winter) typically show increased alkaloid levels (Buirchell personal communication). High rates of N-fixation or high inorganic N fertilization have been reported to increase alkaloid levels (Johnson et al. 1987).

9.1.5.2 Other Antinutritionals

Lupins have several advantages over soybean in relation to antinutritionals. Lupins do not contain lectins (Petterson et al. 1997), while soybean flour has been reported to contain levels of around 100 μg/g and peanuts around 140 μg/g (Sitren et al. 1985). Lectins are heat-labile, and their activity is reduced by cooking. Phytates, which reduce the digestibility of minerals and other nutrients, are low in lupins at 0.5% with levels in soybean reported at 1.0% (Fudiyansyah et al. 1995; Petterson et al. 1997). Saponins in commercial *L. angustifolius* are reported at levels of approximately 570 mg/kg seed (0.057%), which compares with defatted soy flour having 0.58% and navy bean seed with 0.32% (Gurfinkel and Rao 2002). Grains such as quinoa have saponin levels of approximately 0.65%, which necessitate removal prior to consumption (Ward 2001). Total and condensed tannin levels in lupins are reported to be approximately 0.29% and 0.01%, respectively (Petterson et al. 1997), compared with levels in soybean of approximately 0.4%. Condensed tannins are those most responsible for negative effect in protein binding. There is a concentration of the tannins in seed coats of legumes, and dehulling minimizes the quantities in kernels, flour, and meal. Cyanogenic glycosides, which are found in high levels in some legumes such as African yambean, jackbean, several forage legumes, and *Acacia* are present at nil or very low concentrations in lupins.

The major crop lupins contain from 4 to 9% oligossacharides, and these belong to the raffinose family. The major compounds in both lupin and soybean are the galactooligosaccharides, raffinose, stachyose, and verbascose (Hollung et al. 2005; Jiménez-Martínez et al. 2007). Because of their α-1,4 bonding, the oligosaccharides are not metabolized by monogastric animals (pigs, chickens, fish), and therefore, bacterial breakdown occurs in the colon with the subsequent CO_2, methane, and hydrogen gas production. Oligosaccharides, however, have the beneficial effect of osmotic regulation in the gastrointestinal tract (Petterson 1997) and reduce the uptake of normal sugars and cholesterol, thereby having positive effects on body weight. The concentration of oligosaccharide in whole soybean has been recorded from 3 to 5% (Yamka et al. 2005; Wang et al. 2007) to 10% (Hollung et al. 2005). There is scope for reduction of oligosaccharides in legumes through classical breeding and through molecular research with cultivar releases showing reduced oligosaccharides and high protein (Hartwig 1996). It has been shown in soybean that concentrations of carbohydrates were not associated with seed yield, and therefore, selection can occur for lines with improved seed quality and high yield (Wilcox and Shibles 2001). In general, fermentation of legume seeds leads to an improved protein quality and digestibility, increased palatability, and reduction of phytates and oligosaccharides. Many soy products are derived from fermentation, and similar products can be made from lupins.

The cell walls in *L. angustifolius* kernels are composed of non-starch polysaccharides, (lupin kernel fiber or NSP) and form approximately 23% of seed weight. Although this NSP is a valuable dietary fiber (Evans 1994), it can reduce digestible energy in monogastric diets. It may be possible to decrease NSPs either through direct breeding and selection or indirectly through selection for higher protein. In soybean, a strong negative correlation has been reported for seed cell wall polysaccharides and protein plus oil concentration (Stombaugh et al. 2000). Brillouet and Riochet (1983) also found a strong negative correlation between protein plus oil concentration and percentage cell wall material in kernels in various lupin species. *L. mutabilis* is a species with high protein and oil and, as might be expected, has lower NSP levels of around 9% (Brillouet and Riochet 1983). Raw soybean meal contains approximately 20% NSP, and approximately one-third of the NSP are soluble (Hollung et al. 2005). Lupin kernel fiber is a potentially valuable by-product of seed processing as it has been demonstrated to have cholesterol lowering properties, transit time reduction in human digestion, the ability to reduce blood glucose in non-insulin diabetics, reduction of blood pressure, and beneficial effects on stool bulking (Hall et al. 2005a, b; Johnson et al. 2006; Lee et al. 2009).

The consumption of raw and inadequately cooked soybean causes a decrease in protein digestibility and nutritive value and also causes pancreatic hypertrophy. The antinutritional effect is due to trypsin inhibitors and lectins and the compact structure of the native forms of soybean major storage proteins. When trypsin inhibitors are heat-inactivated, lectins, lipoxygenases, and major storage proteins are also denatured (Yuan et al. 2008). In the agriculturally important lupin species, protease inhibitors are at very low levels and of minor importance (Wink 2006). Trypsin and chymotrypsin inhibitor activity in *L. angustifolius* is reported at 0.12 and 0.08 mg/g, respectively. This contrasts with levels of 35 mg/g trypsin inhibitor activity reported for soybean (Yuan et al. 2008).

As with other legumes, lupins produce isoflavonoids in shoots (genistein, 2-hydroxy-genistein, wighteone, and luteone) and roots (as for shoots plus angustons A–C, licoisoflavone, and orobol) (von Baer et al. 2000). These compounds are known for a range of pharmacological benefits such as phytoestrogenic activities, anticancer, antioxidant, and anti-inflammatory characteristics, which offer some benefits in relation to arteriosclerosis and related cardiovascular disease (Wink 2006). Seed isoflavone levels in lupins are considerably lower than in soybean (approximately 50 μg/g in lupin and 1–4 mg/g in soybean) (Wink 2006). Increases in isoflavonones in lupins could enhance marketability and may also confer improved fungal disease resistance in the plant. Highest within plant levels of isoflavones and their conjugates were detected in roots and the lower stem tissue in *L. exaltatus* (Garcia-Lopez et al. 2006), and when lupin and other legume seeds are germinated, isoflavone levels often increase. Elicitors can be used also to boost levels of isoflavones for commercial production.

9.1.6 Nitrogen Fixation

For lupins, N fixation has been reviewed by Howieson et al. (1998), and the potential for increasing N fixation in legumes is addressed by Herridge and Rose (2000). Nitrogen nutrition of lupins occurs both through N_2 fixation and when nitrate or ammonium is assimilated by plant roots. Inside the root nodule, the bacteria (bacteroids) reduce dinitrogen to ammonium, which is secreted to the plant in exchange for a carbon and energy source. Lupins transport most nitrogen in the plant in xylem and phloem mainly as asparagine and smaller amounts of glutamine and are therefore regarded as amide plants cf. ureide production in warm-season legumes (Herridge and Brockwell 1988). This reduction of N takes place in the roots and lower stem (Atkins et al. 1979; Pate et al. 1998).

Lupins have a relatively specific rhizobial requirement and are nodulated mainly by the slow-growing *Bradyrhizobium* sp. *Lupinus* (Jordan 1982), although fast-growing strains associated with lupins have been identified (Miller and Pepper 1988). *Lupinus* has, however, been reported to be a promiscuous host legume that is nodulated by rhizobia with a range of chromosomal genotypes, which could even belong to several species of *Bradyrhizobium* (Perez-Galdona et al. 2004). The initiation of nodulation in lupin differs from many other symbioses in that bacterial cells access the host root through intercellular spaces in the root and entry into root cortex cells without evidence of an infection thread. Recent work with the aid of immunolabeling by Gonzalez-Sama et al. (2004) has described this

process in *L. albus*. The nodules are indeterminate and described as "lupinoid" because they differ from other indeterminate nodule morphologies (Howieson et al. 1998). *Bradyrhizobium* sp. *lupinus* is one of the few of the genus that has a host that is an agricultural legume from a Mediterranean-type climate. Many of the strains of the species also nodulate *Ornithopus*, which include the pasture legume Serradella. The onset of the host-rhizobial symbiosis is mediated by an exchange of diffusible signals such as the bacterial lipooligosaccharides, which condition specificity and act as the inducers of the plant response to the micro-symbiont. Lumichrome is an example of a recently identified signal substance (Matiru and Dakora 2005). The host plant then controls most of the nodulation processes including nodule morphology, efficiency, specificity, and function (Caetano-Anolles 1997).

With the observations in Australia that lupins did not form adequate nodulation on newly established cropping land without inoculation with introduced *Bradyrhizobium* bacteria, it was then shown that rhizobial populations that are found to reside in nodules of crop plants of lupins all originate from Europe (Stępkowski et al. 2005). Lupin crop species have not formed associations with native Australian legume Bradyrhizobial strains. Additionally, the inoculant strains used widely over many years (WU425 and WSM471) were not prevalent in nodules of widely surveyed Western Australian lupin crops in 2005 (Howieson and O'Hara 2008), indicating that background European populations were more persistent. In cropping fields that have not had a lupin or saradella legume host for four or more years, reintroducing inoculant strains of *Bradyrhizobium* sp. *Lupinus* is recommended (Evans 2005). A range of practices to improve nodulation effectiveness in lupin crops have been employed, including various seed coatings, peat and clay-based inoculants, use of nodule increasing bacteria, placement of fertilizer below the seed, and use of strains more tolerant of fungicide seed dressings.

Lupin crops relying solely on symbiotically fixed N frequently produce yields that are similar to high N fertilized crops. Adequate mineral N-fed *L. angustifolius* plants in sand culture had a greater flower number and greater branch growth and biomass compared with N_2-fixing plants, but pod set and seed yields were similar, the N-fed plants having a lower harvest index (Ma et al. 1998). Seed percentage N was similar in both N-fixing and mineral N supplied plants, but shoot percentage N was lower by more than half in the N-fixing plants at final harvest compared with the N-supplied treatment (4.3% N in dry matter). In other work, total N in nodulated lupin plants was generally unaffected by N fertilizer addition (Evans et al. 1987). It is commonly found that applied nitrogen reduces nodule number for lupins and other legumes and factors such as drought stress severely reduces nodulation, which, via reduced N fixation, reduces plant growth (King and Purcell 2005).

N_2 fixation is highly correlated with total biomass production for both crop and pasture legumes. Evans et al. (2001) showed that between 20 and 25 kg shoot, N is fixed for every ton of shoot dry matter produced. This contributes to the higher protein of the lupin seed as well as providing residual N for subsequent crops, particularly cereals and Brassica crops. Estimates of shoot nitrogen generated by lupins depend on the environment, but averages from a range of geographic locations are approximately 200 kg N/ha for *L. angustifolius* and 330 kg N/ha for *L. albus* (Howieson et al. 1998). In cold climates such as Iceland, *L. angustifolius* showed symbiotic N yields as high as 185–212 kg/ha. *Lupinus luteus* was found to have higher N fixing rates than *L. albus* and *L. angustifolius* in inoculated plants in the field in Spain (Chamber and Delgardo 1986). Relating lupins to other crops, a survey of the quantities of N_2 fixed per unit area revealed that the principal crop legumes were ranked in the following descending order: soybean, lupin, field pea, faba bean, common bean, lentil, and chickpea (Unkovich and Pate 2003).

Nitrogen fixation improvement is currently not a part of routine breeding programs, and incorporating selection criteria for it in terms of nodule number or mass, nitrogenase activity and N compounds in vascular tissues into breeding schedules along with the other trait priorities is a difficult task. Marker-assisted selection (MAS) for nitrogen fixation if developed could be integrated into breeding programs that are already practicing MAS for other traits. Variation for strain effectiveness has been reported for crop lupin performance in Australia (Howieson and O'Hara 2008) and in Iceland for *L. nootkatensis* (El-Mayas 1999), and rhizobial strains that adapted to desert environments were identified for lupin and bean for enhanced nitrogen fixation (Pepper 1991).

While some molecular genetic studies have begun in lupins relating to nitrogen fixation (e.g., Macknight et al. 1995; Karłowski et al. 2000; Vincze et al. 2004;

Liu et al. 2005; Wolko et al. 2008), this field of research deserves further attention for lupins.

9.2 Diversity Analyses and Wide Hybridization

9.2.1 Gene Bank Holdings

The importance of collection, conservation, documentation, and evaluation has been recognized for lupin because crosses between domesticated types and wild or landrace types is still seen as an important component of breeding programs for improving disease and pest resistance and tolerance to abiotic stresses in this relatively recently domesticated genus. Human activities have greatly impacted lupin populations, especially in the Mediterranean and North African regions. This has both diminished the occurrence of certain wild lupins and increased the spread of other wild or semi-domesticated types.

In 1926, N. I. Vavilov conducted a germplasm collection trip that included *Lupinus* in northern Africa, Cyprus, Crete, Sicily, Sardinia, Spain, Portugal, France, and Greece. Lamberts (1955) collected *L. luteus* after World War II, but it was not until collections made by J. S. Gladstones that contributions to lupin genetic resources collection and evaluation gained momentum. During the 1980s, lupin germplasm, especially of the Iberian Peninsula and Greece was extensively collected and evaluated by a number of scientists (Clements et al. 2005a). It was also during that decade that improved dissemination of information occurred through the International Plant Genetic Resources Institute (Bioversity, previously IBPGR), which included the publication of lupin descriptors (IBPGR 1981). Additionally, the establishment of the International Lupin Association and the International Lupin Conferences (López Bellido 1991) facilitated the exchange of information and germplasm. Summaries of lupin genetic resources have since been published in international legume conferences (Święcicki 1988) and plant introduction reviews and books (Clements and Cowling 1990; Buirchell and Cowling 1998; Cowling et al. 1998a; Cowling 2001). The Database of European *Lupinus* collections was initiated in 1995, and details of lupin accession holdings in Europe were published with other grain legumes. More recent collections and evaluation studies of lupin germplasm collections include the following: *L. angustifolius* of the Aegean region (Clements and Cowling 1994); *L. albus* from the Azores Islands (Papineau and Huyghe 1992), from Portugal (Neves-Martins 1994), from Spain (De Haro et al. 1982), and from Egypt (Christiansen et al. 1999); *L. albus*, *L. angustifolius* and *L. luteus* from the genebank at the Instituto Superior de Agronomia, Portugal (Pereira et al. 2000); *L. pilosus* (Clements et al. 1996); *L. atlanticus* from Morocco (Buirchell 1992; Cowling et al. 1998a); *L. albus* from Ethiopia (Francis et al. 1997); *L. angustifolius*, *L. albus*, *L. luteus*, *L. cosentinii*, and *L. atlanticus* from Morocco (Alami et al. 2004).

Collections of New World Andean lupins, including *L. mutabilis*, were first made in Peru in the 1970s through the University of Cusco and then involving Universidad del Centro in Huancayo and Universidad de Puno often with support from the IBPGR (now Bioversity). The National Lupin Germplasm Collection of Peru was initiated in Huancayo/INIA (Cowling et al. 1998a). Collection and evaluation in Bolivia was conducted by the Pairumani Research Station and in Ecuador by the Instituto Nacional de Investigación Agropecuarias (INIAP). Evaluation studies or summaries of *L. mutabilis* germplasm include Blanco (1982), Neves-Martins (1994), and Planchuelo (1999).

Current total holdings of *Lupinus* germplasm accessions around the world, according to the IPGRI Directory of Germplasm Collections Database (Bioversity 2009), are estimated to be in the proximity of 40,000. Collections of greater than 100 *Lupinus* accessions are listed in Table 9.1. Gene Banks specializing in the Old World lupins include the Australian Lupin Collection (ALC), Vavilov Institute, Russia; Junta de Extremadura. Servicio de Inv. y Desarrollo Tecnologico Finca la Orden, Guadajira, Spain; Centro de Recursos Fitogeneticos, INIA, Alcala de Henares, Madrid, Spain; Station d'Amélioration des Plantes Fourragères, INRA, Lusignan, France; the Gene Bank, Institute for Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany; the University of Reading, England; the Plant Breeding Station, Wiatrowo, Poland. The Vavilov Institute preserves approximately 30 species of *Lupinus* and has considerable evaluation data available in Russian, although much of it is not published. Substantial collections of *L. hispanicus* are held in Spain such as in the

Table 9.1 World *Lupinus* germplasm collections of ≥100 accessions

Institute country	Institution	Total lupin accessions	*L. albus*	*L. angustifolius*	*L. luteus*	*L. mutabilis*	Other New World	Rough-seeded	Comments
Australia	Australian Lupin Collection, Department of Agriculture and Food Western Australia.	4,665	979	2,165	463	221	54	613	All Old World spp. represented (except *L. mariae-josephi* and *L. somaliensis*). Duplicates held Horsham, Victoria, AUS
Bolivia	Centro de Investigaciones Fitoecogeneticas de Pairumani, Cochabamba	114				114			Landraces
Chile	Campex, Temuco	183	70	4	4	100	4		
	Inst de Inv. Agropecuarias, Centro Regional de Investigacion Carillanca, Temuco	1,259	12						Landraces, many accessions, species not specified
Ecuador	Instituto de Ciencias Naturales Universidad Central del Ecuador, Quito	130				130			
	Estacion Experimental Santa Catalina, DENAREF, INIAP, Quito, Pichincha	547	51	35	10	396	27	3	*L. mutabilis* mainly from ECU, landraces and wild types
France	Institut National de la Recherche Agronomic, Le Rheu	1,100	1,100						
	Station d'Amélioration des Plantes Fourrageres, INRA, Lusignan	2,046	1,398	168	148	332			Cultivated and genetic stock
Germany	Institut fuer Pflanzengenetik und Kulturpflanzenforschung, Gatersleben	1,969	134	404	254	987	39	56	Mix of domesticated, landrace and wild types
	Federal Centre for Breeding Research on Cultivated Plants (BAZ), Research and Coordination Centre for Plant Genetic Resources (FKZPGR), Quedlinburg	1,969	134	404	254	987	39	56	As per Gatersleben
	Stuttgart, State Plant Breeding Institute, University of Hohenheim	200							Breeding lines and inbred lines, species not specified
Hungary	Institute for Agrobotany, Tápioszele	451			1				Species not specified
Italy	Bari	100							Species not specified
Kenya	National Genebank of Kenya, Crop Plant Genetic Resources Centre, KARI, Muguga	101	20	9	6	28	5		
Lithuania	Voke Branch of the Lithuanian Institute of Agriculture, Traku Voke, Vilnius reg.	120							Species not specified
Peru	Estacion Experimental Agraria Banos del Inca, INIEA, Cajamarca	347				347			
	Centro Regional de Investigacion en Biodiverisdad Andina (UNSAAC), Cusco	1,940		60	50	1,800			*L. mutabilis* landraces from BOL, PER
	Universidad Nacional San Antonio Abad del Cusco (UNSAAC/CICA), Cusco	1,800				1,800			*L. mutabilis* landraces from ARG, BOL, COL, ECU, PER
	Estacion Experimental Agropecuaria Santa Ana, INIEA, El Tambo, Huancayo	1,725				1,725			*L. mutabilis* landraces from PER
	Universidad Nacional Agraria La Molina, La Molina, Lima	300				300			*L. mutabilis* landraces mainly from PER
	Estacion Experimental Illpa-Puno, INIEA, Puno	138							Species not specified but possibly *L. mutabilis*
	Universidad Nacional del Altiplano, Puno	319				319			

Poland	Poznań Plant Breeders, Breeding Station Wiatrowo	1,049	249	248	421	14	23	29	Mix of domesticated, landrace and wild types
	Plant Breeding and Acclimatization Institute (IHAR), Radzikow, Blonie	509							Species not specified
	Warsaw 76	150							Species not specified
Portugal	Instituto Superior de Agronomia de Universidade Tecnica de Lisboa, Lisboa Codex	661	377	37	78	149	5	5	Mix of domesticated, landrace and wild types
	Banco de Germoplasma-Genetica, Estacao Agronomica Nacional, Oeiras	855	270	240	210		11		Landrace and wild types
	Seccao de Genetica Estacao Agronomica Nacional, Oeiras	288			288				Mostly landrace and wild types
Romania	Satu Mare	100	100						Cultivars and breeding lines
Russian Federation	N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry, St. Petersburg	2,450	417	650	751	129	301	48	Mix of domesticated, landrace and wild types
Spain	Centro de Recursos Fitogeneticos, INIA, Alcala de Henares, Madrid	1,554	567	537	240	20		13	Mostly landrace and wild types; 177 *L. hispanicus*
	Ramon Batlle Vernis, S.A., Bell-Lloch (Lerida)	254	112			142			Breeding lines and landraces
	Junta de Extremadura. Servicio de Inv. y Desarrollo Tecnologico Finca la Orden, Guadajira	1,804	694	553	353				Especially Spanish *L. albus*, *angustifolius*, *luteus*; 204 *L. hispanicus*
	Granja-Escuela de Capacitacion y Experimentacion Agropecuaria, Huelva	436	15	6	15	400			
	Compania Espanola de Cultivos Oleaginosos, S.A. (CECOSA), Madrid	260							Species not specified, mostly breeding lines
	Departamento de Pastos y Forrajes, Salamanca	749	6	410	80				Wild types and breeding lines; 253 *L. hispanicus* – some "natural hybrids"
Syrian Arab Republic	International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo	180	25	37	3		31		Wild types and landraces
Ukraine	Institute of Agriculture, Kiev	910							Species not specified
United Kingdom	Agric. Botany, Plant Sci. Lab., School of Plant Sc., Univ. Reading, Reading	1,300	1,100			200			*L. albus* from GRC, ITA, PRT, ESP
USA	Western Regional Plant Introduction Station, USDA-ARS, Washington State University, Pullman	1,183	327	187	82	79	373	19	More than 50 different New World species represented
	Desert Legume Programme, Tucson, Arizona	204	12	5	21	8	102	15	
	Gold-Smith Seed Co., Inc., Visalia, CA 93277	435	160	50		200	25		Advanced breeding material and L. succulentus wild types
Totals		36,854	8,329	6,209	3,732	10,927	1,039	857	

Sources: Bioversity Directory of Germplasm Collections (Bioversity International 2009), N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry database

Departamento de Pastos y Forrajes, Salamanca. Significant collections of New World species, notably *L. mutabilis*, are held in South American institutions. The Western Regional Plant Introduction Station, USDA-ARS, Pullman, holds a diverse collection of lupins including more than 50 New World species, and relatively large numbers of American species are held in Gatersleben, Germany, and by the Institute for Agrobotany, Hungary. The Vavilov Institute holds a significant collection of *L. polyphyllus*, *L. mutabilis*, and other New World species. Approximately half of the world's gene bank-preserved *Lupinus* accessions are of the three major domesticated, smooth-seeded species, *L. albus*, *L. angustifolius*, and *L. luteus*, and one quarter are *L. mutabilis*. There are only very small holdings of the rough-seeded species *L. digitatus*, *L. palaestinus*, and *L. princei*. The lesser known, Old World, smooth-seeded species *L. micranthus* is poorly represented, the largest holdings (51 accessions) available from the ALC. The *L. albus* wild form var. *graecus* is also not well-preserved compared with landrace and domesticated accessions of the species (var. *albus*). Although a relatively large number of accessions are conserved for *L. mutabilis*, information on their passport, collection site, and preliminary evaluation requires further development. With the range of geography and microclimates of sites in the region of diversity of *L. mutabilis* and related species, in addition to the start of genetic erosion (Planchuelo 2000), it is of high priority to document existing germplasm collections to determine duplication and gaps in collection regions. Useful internet resources for lupin germplasm include Bioversity Directory of Germplasm Collections, USDA GRIN database, the Eurisco website, and the N. I. Vavilov All-Russian Scientific Research Institute of Plant Industry website.

9.2.2 Diversity Analyses and Germplasm Screening

Several diversity studies have been conducted on lupin germplasm collections, which have provided useful knowledge for crop improvement programs. Simpson (1986a, b), using multivariate statistical methods, usefully characterized four geographical races of *L. albus* in the Mediterranean region. The Balkan group in that study included the wild progenitor var. *graecus* types. Approximately 200 Portuguese *L. albus* ecotypes were evaluated by Neves-Martins (1986, 1994), who described winter, spring, and intermediate types using a range of morphological characters, and this highlighted the existence of the "*megalosperma*" ("lupini" types) with large seeds and vegetative structures that were distributed near Leiria on the central coast.

In *L. pilosus*, and similarly in *L. angustifolius*, Clements et al. (1996) described several groups related to geographic origins using multivariate analyses based on morphological characters. Clements and Cowling (1994) identified useful clusters of Greek accessions with vigorous growth and early flowering, which were then used in crossing programs. Clements and Cowling (1990) summarized the ALC germplasm for collection site data across the Old World species, and more recent statistics are given in Table 9.2. It shows the wide soil pH range and collection site altitude for both *L. albus* and *L. angustifolius*, while *L. luteus* (having a lower average soil pH) is more restricted in terms of altitude, soil pH, and plant traits such as seed size. More complete collection site data for *L. mutabilis* is necessary to assess the full range of germplasm that exists in South American gene banks. Berger et al. (2008a) analyzed bioclimatic variables from *L. luteus* collection sites finding three distinct groupings. European genotypes possessed a range of desirable traits including drought avoidance (through earliness and vigor), while Mediterranean germplasm offered potential drought tolerance traits, if associated with more stressful Mediterranean habitats. Collection sites for *L. angustifolius*, *L. albus*, and *L. luteus* were further studied through multivariate analyses and showed that *L. luteus* germplasm represented the narrowest habitat range compared with the other crop species, which formed more complex groupings covering a broader range of habitats (Berger et al. 2008c). Use of low-copy nuclear genes (Hughes et al. 2006) and mitochondrial RFLP analysis (Olczak et al. 2001) has proven to be valuable tools for taxonomic studies and may also be useful in characterizing the variability among the diverse New World lupins.

Screening for individual traits in lupin germplasm has covered a wide range of traits. A good range of seed protein contrasted a narrow variation range for oil in the germplasm screening of Gladstones and Crosbie (1979) for *L. angustifolius*. Seed coat and pod wall proportion variation was identified, particularly in *L. angustifolius* germplasm (Mera et al. 2004; Clements et al. 2005b), and pod wall proportion was found to be correlated with seed yield in studies by

Table 9.2 Selection of plant and collection site data averages for the Australian Lupin Collection wild and landrace germplasm based on total maximum accessions numbering for *L. albus* $N = 588$, *L. angustifolius* $N = 1{,}179$, *L. luteus* 108, and *L. mutabilis* 21 (Percent dry basis, %db)

	Flowering time (days)	Height 9 weeks (cm)	Height maturity (cm)	100 seed weight (g)	Seed oil (%db)	Seed protein (%db)	Coll. site, altitude (m)	Coll. site, soil pH
L. albus								
Mean	94	24	129	31.4		35.2	792	7.9
Minimum	59	3	40	11.2		31.3	1	5.0
Maximum	151	58	205	77.0		39.4	2,600	9.5
L. angustifolius								
Mean	103	16	90	10.8	4.7	31.5	381	6.7
Minimum	73	3	25	3.0	2.7	18.8	1	4.2
Maximum	130	67	170	23.5	6.5	40.6	1,800	9.0
L. luteus								
Mean	115	12	105	9.7	5.2	36.9	158	6.3
Minimum	76	2	50	5.3	4.5	34.4	10	5.5
Maximum	150	43	150	15.0	5.7	37.5	500	8.5
L. mutabilis								
Mean	92	47	147	18.6	13.6	43.6	2,781	6.8
Minimum	71	19	82	6.8	11.9	38.9	2,200	6.5
Maximum	113	85	205	25.5	17.2	46.7	3,500	7.0

Lagunes-Espinoza et al. (1999). Screening *L. angustifolius* for alkaline soil tolerance in *L. angustifolius* found that soil pH at the collection site was not a reliable predictor of alkaline-induced chlorosis, although significant variation in tolerance was observed (Cowling and Clements 1993). Kerley et al. (2002) evaluated tolerance in Egyptian genotypes of *L. albus* germplasm to limed soils, and Brand et al. (2002) screened rough-seeded lupins (*L. pilosus* Murr. and *L. atlanticus* Glads.) for tolerance to calcareous soils. Disease resistance screening has included anthracnose in several lupin species (Cowling et al. 2000) and in *L. albus* and *L. angustifolius* (Talhinhas et al. 2000; Thomas and Sweetingham 2004), Pleiochaeta brown spot and root rot in *L. angustifolius* (Cowling et al. 1997) and root rot in *L. albus* (Luckett et al. 2008), phomopsis in lupin species (Shankar et al. 1999), and cucumber mosaic virus in *L. angustifolius* (Jones and Cowling 1995).

9.2.3 Interspecific Crossing

With the phylogenetic separation between the western New World (western North and South America), the eastern New World (including east-central parts of South America and southeastern USA), and the Old World species, interspecific crossing attempts between these groups has been difficult. The predominant chromosome number for the western North and South American New World lupins is $2n = 48$, and many attempts have been made to interspecifically cross among the species within this group. An early example was the breeding of the ornamental Russell lupin by horticulturalist George Russell during the 1930s in England. With his aim of selecting brighter flower colors, Russell carried out crosses (without embryo rescue techniques) between North, Central, and Southern American species. Cross combinations from published reports for New World species that were apparently successful include *L. elegans* × *L. mutabilis* viable seeds; *L. pubescens* Benth. × *L. mutablis*, F_1 seeds; *L. nanus* Benth. × *L. mutablis* F_1 seeds; *L. polyphyllus* × *L. mutabilis* viable plants, use of backcrossing to *L. mutabilis*; *L. hartwegii* (synonymous with *L. mexicanus*) × *L. mutabilis* viable seeds; *L. mutabilis* × *L. hartwegii* embryo rescue hybrid F_1 plants, F_2 seed (Clements et al. 2005a). As a first step to broadening the genetic base available to a new *L. mutabilis* breeding program in Australia, crosses were attempted between *L. mutabilis* and a number of New World species ranging in their relatedness to *L. mutabilis* (Clements et al. 2008). Successful combinations, some requiring embryo rescue include *L. mutabilis* × *L. mexicanus*, *L. mutabilis* × *L. hartwegii*, *L. mutabilis* × *L. arizonicus* (S. Watson) S. Watson, and *L. mutabilis* × *L. tomentosus* DC.

Some additional combinations among those species were also successful. Valuable traits such as drought tolerance, broader adaptation, and higher yield could be introgressed into *L. mutabilis* using a potentially large number of New World species. Perenniality can also be transferred to *L. mutabilis* from *L. polyphyllus* to create new forage types with low alkaloid (Kurlovich et al. 2008). Using phylogenetic trees as a guide, further wide crossing within the New World lupins should generate an enormous genetic diversity for a very wide range of applications. Development of ornamental lupins would further benefit in terms of form, color, and scent variation from new interspecific combinations among New World species.

Because the Mediterranean crop lupin species are a much less homogeneous, more widely separated group, interspecific crossing among them or between them and the rough-seeded species or the New World species has been difficult. Apart from *L. luteus* × *L. hispanicus* referred to earlier (Święcicki et al. 1999; Naganowska and Ładoń 2000), reports of confirmed hybrids are rare. Kasten et al. (1991) in Germany rescued embryos of *L. angustifolius* × *L. luteus* and obtained a few isozyme-confirmed hybrid F_1 plants, which when transferred to soil, died at 12 weeks of age. Using modified embryo rescue methods, F_1 hybrid plants confirmed (using resistance gene analog polymorphism markers) between *L. angustifolius* and *L. luteus* have been recently obtained in Australia (Clements et al. 2009). The plants with reduced pollen fertility are flowering, and backcrossing attempts are currently underway. This represents an opportunity to transfer highly complementary traits between these two Old World species including higher protein and sulfur amino acids from *L. luteus* introgressed into *L. angustifolius*.

Some potential was demonstrated by Przyborowski and Packa (1997) for crossing *L. albus* × *L. angustifolius* via embryo rescue at 15–20 days after pollination, although confirmation regarding the hybrid status of surviving plants was still pending. As an example of crossing between the New World and the Old World group of species, Sawicka-Sienkiewicz and Brejdak (1999) in Poland obtained putative hybrids between *L. mutabilis* and *L. albus*, and some cytological and morphological evidence supporting their hybrid status was obtained (Sawicka-Sienkiewicz et al. 2008). From phylogenetic studies, this combination is more likely to succeed than other combinations between the New and Old World species.

Interspecific crossing among the rough-seeded lupins has been reasonably successful with fertile hybrids now fully domesticated (Gupta et al. 1996). This is despite their range of chromosome numbers, and the compatibility is likely due to their very homogeneous phylogeny. The most mutually compatible species in crossability studies were the groups *L. cosentinii* ($2n = 32$), *L. digitatus* ($2n = 36$), and *L. atlanticus* ($2n = 38$). Crosses between *L. pilosus* and *L. palaestinus* (both $2n = 42$) have been achieved. Introgression among the rough-seeded lupins should allow the transfer of domestication traits (reduced shattering, permeable seeds, early flowering) and adaptation to fine textured, neutral to alkaline soils and tolerance to free lime. In several two- and three-way crosses among *L. cosentinii*, *L. digitatus*, and *L. atlanticus*, a range of univalent frequencies were observed, the highest being between *L. cosentinii* and *L. digitatus*, and usually an intermediate chromosome number resulted.

The breeding efforts reported in Roy and Gladstones (1988), Buirchell and Cowling (1992), and Gupta et al. (1996) have allowed the transfer of important domestication genes from *L. cosentinii* and *L. digitatus* into *L. atlanticus*. Seed permeability, early flowering, and one of the genes (*ma*) coding for non-shattering pods were transferred to *L. atlanticus* type plants. These hybrids had chromosome numbers varying from 34 to 38. Of the two non-shattering genes, only *ma* has been found to be expressed in hybrids with *L. atlanticus* and only where *L. digitatus* was in the pedigree. A hybrid between *L. atlanticus* and *L. digitatus* has produced an early flowering hybrid, which is thermoneutral. Neither of the parents exhibits any of the early flowering characteristics expressed by this hybrid. The earliness is controlled by a single dominant gene. Through interspecific crossing, *L. atlanticus* is now considered to be fully domesticated, combining low alkaloids, non-shattering pods, and permeable seed coat, the latter coming from *L. cosentinii* cv. Erregulla-soft.

9.3 Cytogenetics and Biotechnology

9.3.1 Genome Size

Nuclear DNA content variation is a key diversity character in systematic and phylogenetic studies in plants (Bennett and Leitch 2005). Complemented

with information at other levels of plant organization, it helps to elucidate interspecific relationships. Flow cytometry, a fast and accurate method, is being widely applied for the estimation of plant genome size.

Measurements of the nuclear DNA content in *Lupinus* species were reported by a few authors. Bennett and Smith (1976) published 1C values for *L. albus* (0.60 pg), *L. luteus* (1.00 pg), and a New World species *L. arboreus* (0.90 pg). Barlow (1981) gave 1C for *L. angustifolius* as 0.93 pg. Obermayer et al. (1999) analyzed *L. anatolicus*, *L. pilosus*, *L. luteus*, *L. hispanicus* ssp. *hispanicus*, and a hybrid *L. hispanicus* ssp. *hispanicus* × *L. luteus* and determined 1C values ranging from 0.60 pg in *L. anatolicus* to 1.17 pg in *L. luteus*. Gammar et al. (1999) studied variation in nuclear DNA content within and between populations of eight Old World lupins but did not present absolute DNA amounts. According to Bennett and Leitch (2005), those data should be treated with caution unless confirmed independently as they differ greatly from earlier measurements. Hajdera et al. (2003) reported on 2C values for *L. angustifolius* and *L. cosentinii* as 2.07 pg and 1.54 pg, respectively. Several studies concerned the level of endopolyploidy in *L. albus* (Le Gal et al. 1986), *L. luteus* (Olszewska and Legocki 1989; Sakowicz and Olszewska 1997), and *L. angustifolius* and *L. cosentinii* (Hajdera et al. 2003).

The first study of nuclear DNA content in *Lupinus*, based on data for a large number of taxons, aimed to determine the range of genome size variation and to investigate relationships among the various taxa within the genus, was published by Naganowska et al. (2003b; 2006). The 2C values were estimated by flow cytometry, using propidium iodide as a fluorescent dye, in 18 species and botanical forms from the Old World (all herbaceous, annuals). The lupins represented distinct infrageneric taxonomic groups and differed in somatic chromosome numbers. Genome size displayed significant variation between species (more than 2.5-fold) as estimated 2C DNA values ranged from 0.97 pg (*L. princei*) to 2.44 pg (*L. luteus*). The variation among rough-seeded lupins was 1.7-fold, whereas among smooth-seeded species – 2.5-fold. No significant correlation between DNA content values and somatic chromosome numbers was found. Statistical analysis resulted in "homogeneous groups" that reflected the generally accepted taxonomic classification of the Old World lupins, i.e., obtained groups coincided with *Lupinus* sections. The rough-seeded *L. princei*, with the lowest 2C value, was an interesting exception as it got closer to smooth-seeded species. The analysis confirmed the heterogeneous character of the smooth-seeded group that might be a result of several independent evolutionary lineages from the ancient rough-seeded stock.

Similar analysis of the DNA content values, based on data for 38 species and accessions, was published for the New World lupins (Naganowska et al. 2006). Studied taxons were annuals and perennials, representing different taxonomic "complexes" proposed by Dunn (1984). Out of lupins analyzed, only genome size of *L. arboreus* was previously measured (Bennett and Smith 1976). Estimated 2C DNA values ranged from 1.08 pg in *L. pusillus* to 2.68 pg in *L. albicaulis*, both from North-America, so the 2.5-fold overall variation was similar to that of Old World species. The variation for North American lupins was much higher than that for South American ones. North American species clearly grouped according to their annual or perennial life cycle (annual species having smaller genomes than perennials). Preliminary data suggest that no correlation exists between 2C values and chromosome numbers in North American lupins. The poorly defined taxonomy and the limited sampling from South America did not allow the investigation of different aspects of interspecific relationships among the New World taxa.

Species of the genus *Lupinus* are characterized by small genomes, only a few times larger than that of *Arabidopsis thaliana*, the model plant genome. Slight differences in measurements reported for individual species by different authors may not only be due to the origin of the material but also to the various internal standards used for flow cytometry. Studies on New World lupins need to be continued for a larger number of species to obtain complete pattern of interspecific relationships. Data on nuclear DNA content can be continuously verified based on the DNA content databases developed for Angiosperms, open for researchers, currently complemented with new results (*http://www.kew.org/cval/homepage*; Bennett and Leitch 2003, 2005).

9.3.2 Chromosome Number

Lupinus species are considered to be paleopolyploids (Atkins et al. 1998; Gladstones 1998), and thus

in their early evolution, the events of allo- and autopolyploidization probably occurred, followed by genome divergence and the "diploidization" process (Wendel 2000). Contemporary forms studied are mostly functional diploids; however, the ploidy level of lupins is not clear and the great diversity in chromosome numbers is difficult to explain. Lupin chromosomes are generally small and similar in morphology. Cytological preparations of good quality are difficult to obtain; however, chromosome countings in lupins have been performed for over 60 years (see Atkins et al. 1998 for a review). Two main groups of species, from the Old and New World, differ in many aspects, including cytological characteristics.

The Old World lupins, a small group of multifoliolate annuals, mostly autogamous, reveal a series of different basic chromosome numbers ($x = 5$, 6 or 9, 7, 8 and 13) as well as various somatic numbers (Pazy et al. 1977; Atkins et al. 1998). Chromosome numbers of the rough-seeded species range from $2n = 32$ for *L. cosentinii* and $2n = 36$ for *L. digitatus* to *L. atlanticus* and *L. princei* with $2n = 38$ and *L. pilosus* and *L. palaestinus* both having $2n = 42$. For smooth-seeded Old World lupins, they are higher, ranging from $2n = 40$ in *L. angustifolius* to $2n = 50$ in *L. albus* and $2n = 52$ in *L. micranthus*, *L. luteus*, and *L. hispanicus*. A few meiosis analyses were performed, showing mostly regular meiotic behavior in the Old World species (Pazy et al. 1977; Carstairs et al. 1992). Some measurements of mitotic chromosomes for several Old World species were made (Pazy et al. 1977; Carstairs et al. 1992; Naganowska and Ładoń 2000). A first complete karyotype was set for *L. angustifolius* (Kaczmarek et al. 2009) based on computer measurements of mitotic metaphase chromosomes and the use of molecular cytogenetic markers. The narrow-leafed lupin chromosomes are meta- or submetacentric. The mean absolute chromosome lengths ranged from 1.9 μm to 3.8 μm, and mean relative lengths from 1.6% to 3.3%.

Cytological data on American species are limited. In contrast to the Old World, a very large group of lupins from the New World, including several hundred multifoliolate or unifoliolate (considered as more primitive) species, herbaceous or shrubby, annual, biennial, or perennial species, reveals mainly the basic chromosome number $x = 6$ and a low variability of somatic chromosome numbers (Dunn 1984; Gladstones 1998; Conterato and Schifino-Wittmann 2006).

Most of the North American species studied, as well as some Andean ones (*L. microphyllus*, *L. mutabilis*, *L. paniculatus* and *L. pubescens*), have $2n = 48$, and only occasionally 96 or others (see for review, Gladstones 1998). Recent studies on several multifoliolate Andean taxa (*L. ballianus*, *L. bandelierae*, *L. eanophyllus*, *L. huaronensis*, and *L. semperflorens*) and eight taxa from Peru and Bolivia not yet formally named determined the same chromosome number $2n = 48$ (Conterato and Schifino-Wittmann 2006). These data support an earlier conclusion on close relationship between Andean lupins and the North American ones. One exception was found – an Andean species *L. bandelierae* with $2n = 36$ (Conterato and Schifino-Wittmann 2006); however, its geographical distribution suggests affinities rather with species from further south. On the other hand, for two unifoliolate North American lupins *L. cumulicola* and *L. villosus*, the chromosome number $2n = 52$ was stated (Conterato and Schifino-Wittmann 2006). It was not reported for any American species before but is characteristic for three Old World smooth-seeded lupins.

In southeastern South American lupins, a group including multi- and unifoliolate species, chromosome number $2n = 36$ was found as a rule, i.e., for seven multifoliolate species (*L. gibertianus*, *L. lanatus*, *L. magnistipulatus*, *L. multiflorus*, *L. rubriflorus*, *L. reitzii*, *L. uleanus*) (Maciel and Schifino-Wittmann 2002), then for multifoliolate *L. paraguariensis* and *L. paranensis*, as well as for unifoliolate *L. crotalarioides*, *L. guaraniticus*, and *L. velutinus* (Conterato and Schifino-Wittmann 2006). Only *L. bracteolaris* and *L. linearis* both had $2n = 34$ and 32, respectively (Maciel and Schifino-Wittmann 2002). First data on meiosis for several Brazilian species were presented as well, showing meiotic stability (Conterato and Schifino-Wittmann 2006).

Chromosome analyses of American *Lupinus* sp. suggest that southeastern South American species are cytologically differentiated from the Andean as well as from most other American lupins. The difference in chromosome numbers between the two unifoliolate groups is an argument for independent origins of the Brazilian and the North American unifoliolate species. Understanding of relationships among different taxa within the genus needs further cytological investigations; however, chromosome analyses should be integrated with genome analysis by molecular cytogenetics methods and with phylogenetic studies.

9.3.3 Cytogenetic Mapping

Cytogenetic mapping has not been extensively performed in *Lupinus* and concerned mostly the Old World species. The application of the principal method of molecular cytogenetics, fluorescence in situ hybridization (FISH), has provided potential insight into the genome structure by localization of DNA sequences in chromosomes. FISH is a method based on complementary binding of a molecular probe (a labeled DNA fragment) in chromosomes, directly on a cytological preparation, and observation of signals under a fluorescence microscope. The use of FISH in chromosome studies relies on the availability of suitable DNA markers. Clusters of rRNA genes make suitable targets for initial studies on physical genome mapping in plants. As the first step in cytogenetic mapping in lupins, genomic distribution of rDNA was determined by double-target FISH, altogether in 12 Old World species and one New World species *L. mutabilis* (Naganowska and Zielińska 2002; Hajdera et al. 2003; Naganowska et al. 2003a; Naganowska and Kaczmarek 2005). Sequences of rDNA (5S and probes based on conserved fragments of 45S rDNA) were mapped at only few discrete chromosomal sites, permitting unambiguous identification of two to five different pairs of chromosomes, depending on the species. Thus, they have been of limited value as chromosome markers. No correlation between numbers of rDNA loci and chromosome numbers was observed in the studied lupins. It needs to be stressed that in all 12 species, one locus of 45S rDNA gave an exceptionally large FISH signal in one chromosome pair, covering the major part of the chromosome, including the nucleolar organizer region (Fig. 9.3a). The large size of that signal, as well as low rDNA loci numbers in general, seems to support the widely accepted idea that polyploidy in *Lupinus* is of ancient origin, since during the long diploidization process, some rDNA sites might be lost (Thomas et al. 1997) or translocated and fused with other rDNA sequences, as it is suggested for *Brassica* (Snowdon et al. 1997). Some analyses of nucleolar activity were also conducted in *L. luteus* (Sakowicz and Olszewska 1997), *L. angustifolius*, and *L. cosentinii* (Hajdera et al. 2003).

More studies on cytogenetic mapping were performed for the narrow-leafed lupin (*L. angustifolius*).

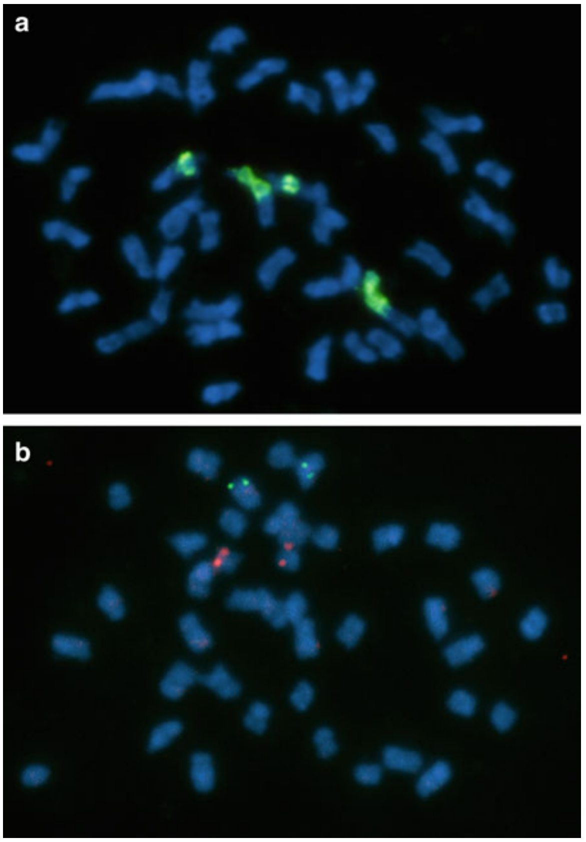


Fig. 9.3 Mitotic metaphase chromosomes of *Lupinus angustifolius* with signals of FISH reactions: (**a**) rDNA (*larger green signals*) and the BAC clone including three markers for disease resistance (*smaller signals*); (**b**) two clones randomly chosen from the BAC library (*green* and *red signals*) (photograph by K. Leśniewska)

Besides rDNA, some tandemly repeated sequences were localized by FISH (i.e., telomere sequences), but still a relatively small proportion of chromosomes could be identified (Hajdera et al. 2003; Naganowska and Kaczmarek 2005). Primed in situ DNA labeling (PRINS) and its variant cycling PRINS (C-PRINS), alternative methods of molecular cytogenetics, which were applied to *L. angustifolius*, contributed to the lupin chromosome marker pool. They allow the detection of shorter DNA sequences by amplification of DNA fragments using sequence-specific primers, directly in chromosomes, and observation of signals with fluorescence microscope. Some oligonucleotide sequences have been localized and facilitated the identification of several chromosome pairs (Kaczmarek et al. 2007). Chromosome-specific markers obtained

by methods of molecular cytogenetics contributed to the *L. angustifolius* idiogram (Kaczmarek et al. 2009).

Lately, cytogenetic mapping of *L. angustifolius* has been focused mainly on the exploitation of a bacterial artificial chromosome (BAC) library of the *L. angustifolius* nuclear genome (Kasprzak et al. 2006). BAC clones have been used as molecular probes for FISH (BAC-FISH) and localized in chromosomes. Some BACs selected from the library included markers for valuable traits, e.g., disease resistance. Cytogenetic markers for specific chromosomes were developed from physically mapped BACs that generated unique single locus FISH signals (Fig. 9.3b). Then sequencing of some BAC ends made it possible to generate markers for genetic mapping. Integration of these approaches allowed the assignment of a number of linkage groups to particular chromosomes (Leśniewska et al. 2009). This work is in progress and provides the foundation for a complete integration of linkage groups from the genetic map with the narrow-leafed lupin karyotype.

9.3.4 Biotechnology

Development of cell and tissue culture techniques as well as genetic manipulation methods has opened up new prospects for breeding by plant transformation and by releasing from transformants novel genotypes of increased value. The transformation process is aimed at delivering new genes by introducing foreign DNA into the genome. It requires a source of totipotent cells and a means of delivering the DNA. In vitro systems of plant regeneration are the main source of target cells. Two systems of DNA delivery are currently being used: the first is based on infection by *Agrobacterium tumefaciens* or by *A. rhizogenes*, and the second is based on the direct introduction by microinjection, electroporation, or microprojectile bombardment. For selecting transformed cells, several marker genes are exploited, mainly those associated with herbicide or antibiotic resistance.

Possibilities of increasing the variability in lupins by interspecific hybridization are limited due to strong incompatibilities within the genus. Nevertheless, lupins are not extensively used in biotechnological programs in comparison to other Fabaceae crops. Like most large-seed legumes, they are generally recalcitrant to tissue culture manipulation and transformation; moreover, their regeneration in vitro is highly genotype-specific and requires considerable technical expertise. Progress in *Lupinus* biotechnology is still limited and in fact concerns only a few species. However, a range of protocols for tissue cultures from various explants have been reported for lupins, with various degrees of regeneration success (for review see Atkins and Smith 1997; Święcicki and Święcicki 2000). Procedures included callus production (for *L. albus*, *L. angustifolius*, *L. hartwegii*, *L. luteus*, *L. polyphyllus* – Sator 1985; Sroga 1987; for *L. mutabilis* – Phoplonker and Caligari 1993) or direct development from explants (for *L. luteus* – Daza and Chamber 1993; for *L. albus*, *L. angustifolius*, *L. hispanicus*, *L. luteus*, *L. polyphyllus* – Rybczyński and Podyma 1993a; for *L. albus*, *L. hispanicus*, *L. mutabilis* – Zgagacz and Rybczyński 1996). Recently, Pniewski et al. (2002) published complete protocols for long-term micropropagation of several cultivars of four lupin crops: *L. luteus*, *L. albus*, *L. angustifolius*, and *L. mutabilis*. Procedures for these included in vitro shoot regeneration by axillary bud development, multiplication of plantlets, followed by rooting or grafting.

Somatic embryogenesis process was also reported for *L. albus*, *L. angustifolius*, and *L. mutabilis* (Nadolska-Orczyk 1992); procedure for *L. albus* resulted in receiving plantlets (Rybczyński and Podyma 1993b). As embryo rescue culture might provide new possibilities for improving lupin cultivars, a number of modifications of immature embryo cultures aimed at obtaining interspecific hybrids, using various media types and composition, were published for several lupin species (i.e., Vuillaume and Hoff 1986; Podyma et al. 1988; Schäfer-Menuhr et al. 1988; Kasten et al. 1991; Kasten and Kunert 1991; Przyborowski et al. 1996; Clements et al. 2009). However, androgenesis, playing an important role in genetic manipulations in various crops, is of limited use in lupins (see for review, Święcicki and Święcicki 2000). On the other hand, protoplast isolation and somatic hybridization, with the aim of producing interspecific hybrids, have been explored in several lupin species (Schäfer-Menuhr 1990, 1991; Wetten et al. 1999; Babaoglu 2000). Lately, Sinha et al. (2003/2004) optimized protoplast production in *L. albus* and obtained a reproducible yield of protoplasts with more than 70% viability.

In spite of difficulties in genetic manipulations in *Lupinus* species, a number of successful transformations

have been reported (see for reviews, Atkins and Smith 1997; Święcicki and Święcicki 2000; Somers et al. 2003). As one of the first results, hairy root lines of *L. albus* and *L. polyphyllus* were obtained by infection with *Agrobacterium rhizogenes* (Mugnier 1988). Later, Smith et al. (1996) reported on routine transformation of *L. angustifolius* by infection of shoot apices with *A. tumefaciens*. Current strategies of genetic manipulations are aimed at exploiting transformation to improve yield potential through the introduction of valuable genes, using mainly *A. tumefaciens*-mediated gene transfer by inoculation of meristem explants and regeneration of transgenic plants. A series of key reports were published, concerning mainly *L. angustifolius*; thus, new traits were introduced, such as viral disease resistance (Jones 1996), herbicide resistance (Somsap et al. 1994; Pigeaire et al. 1997), and increased seed value by seed composition modifications (Molvig et al. 1997). Li et al. (2000) published the first report on the generation of transgenic plants of yellow lupin (*L. luteus*), a species considered as more difficult to transform than the narrow-leafed lupin; they obtained plants with herbicide resistance introduced by the *A. tumefaciens*-based procedure. As a basis for investigations of oral vaccines, an improved protocol of *L. luteus* seedling and hypocotyl transformation has been worked out, for the induction of transgenic calli or tumors producing a small surface antigen of hepatitis B virus (Pniewski et al. 2006). In white lupin (*L. albus*), an *A. rhizogenes*-based transformation system was recently developed for transgenic root formation, which is connected with *L. albus* tolerance to phosphorus deficiency (Uhde-Stone et al. 2005).

Direct DNA transfer methods have also been used in lupins (e.g., Guines et al. 1999), but they are of limited importance because of their difficulty. Compared with microprojectile bombardment, electroporation, or microinjection, the use of *A. tumefaciens* for DNA transfer in crop genetic engineering is still increasing. Apical meristems are successfully used as sources of totipotent target cells. Application of highly virulent strains and binary vectors, matching *Agrobacterium* strains with plant genotypes, as well as optimization of new selectable markers are important factors for improving transformation efficiency. Simple and reliable transformation systems, with low frequencies of abnormalities, are essential for progress of genetic manipulations in lupins. Thus, probably the systems of non-tissue culture transformation would be a future way to transgenic *Lupinus* plants and should provide transformation efficiency required for functional genomics of lupins.

9.4 Genetic Mapping and Synteny with Model Plants

9.4.1 Genetic Linkage Maps

Genetic linkage can be defined as the tendency of two gene or marker alleles to be transmitted together to the next generation due to their proximal location on the same chromosome. A genetic map of a whole genome can be constructed based on the patterns of allele transmission of many genes or markers distributed across the genome, normally assayed in a controlled population or populations. The first genetic linkage maps for plant species were made for maize and tomato (MacArthur 1934; Emerson et al. 1935) and were based on morphological traits controlled by single genes. These early maps required the synthesis of many separate linkage studies and so whole genome linkage mapping did not become common until the advent of abundant and polymorphic DNA markers in the 1980s. Fittingly, the plant species at the forefront of these developments were again tomato and maize, for which restriction fragment length polymorphism (RFLP) linkage maps were constructed by Bernatzky and Tanksley (1986) and Helentjaris et al. (1986).

9.4.1.1 Linkage Mapping in *L. angustifolius*

To our knowledge, the first linkage mapping experiment in a lupin species began at the Department of Agriculture and Food Western Australia (DAFWA; Perth, Australia) in 1997 with the crossing of a domesticated breeding line (83A:476) and a wild accession from Morocco (P27255) of *L. angustifolius* (narrow-leafed lupin) (Nelson et al. 2006). These two crossing parents differed in all six key domestication traits used in Australian cultivars: *Ku* (early flowering); *iuc* (low seed alkaloid); *ta* and *le* (pod shatter resistance); *moll* (water permeable seed); and *leuc* (color in flowers, seeds, and cotyledons). The first partial linkage map was constructed in the F_2 generation using amplified

fragment length polymorphism (AFLP) markers (Brien et al. 1999). By a process of single seed descent, a population of F_8/F_9 recombinant inbred lines (RILs) was developed and formed the basis of more complete mapping of the *L. angustifolius* genome by Boersma et al. (2005) and Nelson et al. (2006).

The Boersma et al. (2005) map comprised 522 microsatellite-anchored fragment length polymorphism (MFLP) markers in 21 major linkage groups using 89 RILs. The Nelson et al. (2006) map comprised 382 mainly gene-based sequence tagged site (STS) markers in 20 major linkage groups using 93 RILs. The advantage of the arbitrary primer-type MFLP marker system used by Boersma et al. (2005) was that many marker loci were generated with great rapidity. However, MFLP markers are generally cross-specific and are, therefore, of limited value outside the mapping population unless they are first converted to locus-specific STS markers (e.g., Yang et al. 2004). In contrast, the STS markers produced by Nelson et al. (2006) were fewer in numbers but had the advantage that they were applicable across the legume family (see Sect. 9.4.2 below).

The complementary strengths of these two published *L. angustifolius* maps were combined recently in a map incorporating these loci along with an additional 200 new markers genotyped in up to 106 RILs (Nelson et al. 2010). This combined map comprised 1,080 loci in 20 linkage groups (matching the haploid chromosome number for *L. angustifolius*, $n = 20$) ranging in length from 69.7 to 168.1 cm, with a total map length of 2,361.8 cm. This map has recently improved with the addition of 300 diversity array technology (DArT) STS marker loci (Nelson unpublished data).

The combined map was used to locate the last domestication trait to be mapped: *Ta*, one of the genes controlling pod shatter resistance. Boersma et al. (2009) mapped *Ta* to linkage group NLL-01 of the combined map (Nelson et al. 2010), which is equivalent to linkage group 1 (LG1) of Boersma et al. (2005) and LG18 of Nelson et al. (2006). Three markers closely linked to *Ta* were developed, with at least one marker being informative in 70% of all possible crosses between wild and domestic lines screened in that study.

Only one quantitative trait loci (QTL) mapping exercise has been reported for *L. angustifolius* by Boersma et al. (2008). QTLs were identified for early vigor, height, flowering time, and seed size. Interestingly, the genomic region harboring *Ku* had a role in all four traits measured. The connection between slow growth and vernalization requirement in *L. angustifolius* was also commented upon by Berger et al. (2008a) in a comparison of Australian narrow-leafed lupin varieties. Further investigation will be required to determine if these two traits are controlled by the same gene or by closely linked genes.

Another RIL population comprising 260 RILs was developed at DAFWA (Perth, Australia) from a cross between two domesticated Australian varieties of *L. angustifolius*: Unicrop and Tanjil. In this population, there is segregation for resistance to anthracnose disease, cucumber mosaic virus (CMV), seed transmission, aphids, and phomopsis stem blight. Yield data at three sites over two years have also been collected (Buirchell personal communication). This population was used to identify a major gene for anthracnose disease resistance (*Lanr1*) and to develop a marker for marker-assisted selection (Yang et al. 2004).

While the Unicrop × Tanjil mapping population is segregating for a number of important traits, it suffers from very low frequencies of marker polymorphism, estimated to be approximately tenfold less than in the wild × domestic RIL population (Nelson unpublished data). This low level of DNA polymorphism will make map construction relatively inefficient using conventional marker technologies.

9.4.1.2 Linkage Mapping in *L. albus*

Two linkage maps have been developed for *L. albus* (white lupin) based on independently developed RIL populations in Australia and the UK. Phan et al. (2007) used 105 STS and 220 AFLP markers to genotype 94 F_8 RILs (randomly selected from a larger population of 195 RILs) from a cross between the cultivar “Kiev Mutant” and an Ethiopian landrace “P27174.” Twenty-eight major linkage groups were identified, which exceeds the haploid chromosome number for *L. albus* ($n = 25$), indicating that this map did not adequately cover at least three chromosomes. QTLs were identified for anthracnose resistance and flowering time, and a single major gene for seed alkaloid content was also mapped.

An independent mapping study was performed on a population of 102 F_5 RILs (from a larger population of 115 RILs) developed from a cross between "Lublanc" (a spring cultivar) and "LD37" (a graecus type). Using 77 STS and 230 AFLP markers, 25 linkage groups were identified, which the authors estimated to represent 84.2% genome coverage (Croxford et al. 2008). QTLs were identified for flowering time, seed alkaloid content, and stem height. Only a few markers were shared between the two studies, making comparisons between maps difficult.

9.4.1.3 Linkage Mapping in Other Lupin Species

Maps for other lupin species have not yet been reported. However, work is underway in Chile (Biotechnology Unit, INIA-CGNA) to develop the first linkage map of *L. luteus* (yellow lupin; Maureira Butler and Salvo-Garrido personal communication). In Australia, two populations, each consisting of 200 F_8 RILs, have been developed for *L. luteus* (Adhikari personal communication). One population (from a cross between two domesticated parents) is segregating for a single dominant gene for CMV resistance. The other population (from a cross between wild and domesticated parents) is segregating for seed coat permeability, flowering time, alkaloid content, seed coat color, pod shattering, and flower color. There are no immediate plans to develop genetic maps using these populations.

9.4.2 *Synteny and Colinearity of Lupin Genomes*

"Synteny" (from the Greek word for "same thread") in its original genetic meaning simply refers to the co-location of loci on the same chromosome (Passarge et al. 1999). However, the term synteny appears to be subject to its own rapid evolutionary process whereby it has taken on a second meaning of "conserved co-location of loci between two related organisms" and then a third meaning of "conserved order of loci between related organisms." In the hope of reducing confusion over terminology, we use synteny in its second meaning (conserved co-location of loci) and use the term "colinearity" to refer to conservation of locus order.

9.4.2.1 Synteny in the Legume Family

Comparing the genetic maps of related species can shed light on genome evolution such as the occurrence of chromosomal translocations, inversion, fusions, and fissions. In plant species, the earliest studies were in the Poaceae and Brassicaceae families (Moore et al. 1995; Lagercrantz and Lydiate 1996), but more recently, this approach was applied to galegoid and phaseolid clades (containing most agricultural legume species) of the Fabaceae family (Choi et al. 2004). Chromosomes within the galegoid clade appeared, in general, to be well-preserved with more differences between the galegoid and phaseolid clades.

9.4.2.2 Synteny Between Lupin Species and the Model Genome *Medicago truncatula*

Since the first legume analysis, synteny analysis has been extended to the genistoid clade with comparison of the lupin species *L. angustifolius* and *L. albus* to the model legume species, *M. truncatula* (Nelson et al. 2006; Phan et al. 2007), and comparison of *L. angustifolius* to the genome sequence of *Lotus japonicus* (Nelson et al. 2010; Sato et al. 2008). These studies revealed more frequent rearrangements and breakages of synteny between the lupin species (genistoid clade) and *M. truncatula* and *L. japonicus* (both galegoid species) compared to that seen within and between the galegoid and phaseolid clades (Choi et al. 2004), and more even than between galegoids and the relatively basal dalbergoids as represented by the genus *Arachis* (Bertioli et al. 2009). These results supported phylogenetic analysis of Lavin et al. (2005) that estimated that the genistoid clade branched off from the other legume clades around 56.4 million years ago, considerably earlier than the galegoid and phaseolid clades (part of the IRLC crown node) that branched off 39.0 million years ago. However, it is less clear why *Lupinus* has diverged more markedly from those clades than *Arachis*, which belongs to the dalbergoid clade that diverged from the other legume clades at a similar time to genistoids (55.3 million years ago; Lavin et al. 2005).

It should be noted that the relatively low densities of gene-based STS markers identifying orthologous sequences in the model legume genomes [140, 97 and 171 such "bridging points" in Nelson et al.

(2006), Phan et al. (2007), and Nelson et al. (2010), respectively] make the resolution of these lupin synteny analyses rather low. As more STS markers are added to the lupin maps, more fine-scale comparisons to sequenced legume genomes will become possible. Furthermore, with the recent release of the soybean genome sequence (Schmutz et al. 2010), comparison of a lupin genome map to a phaseolid genome sequence is now possible and would shed further light on legume genome evolution.

9.4.2.3 Synteny Among Lupin Species

Lupin species show a range of chromosome numbers (Naganowska et al. 2003b; 2006). It would be interesting to use synteny analysis to investigate how those changes in chromosome numbers occurred and to infer the chromosome number of the founding ancestor of the genus *Lupinus*. As described above, linkage maps based on cross-species STS marker have been developed for *L. angustifolius* and *L. albus*: Do these shed light on the comparative genome structure of these lupin species?

The most basic form of synteny that can be described involves two loci being found on a single chromosome in both species. Phan et al. (2007) reported just two examples of this when comparing their map of *L. albus* with the map of *L. angustifolius*. We reanalyzed the two maps, and among the 26 marker loci mapped in both species, we found four pairs of syntenic loci, which are listed in Table 9.3. There were nine other pairs of loci that were located on one linkage group in one species but on two separate linkage groups in the other species (i.e., synteny could have been observed but was not; data not presented). We carried out a similar comparison of *L. angustifolius* and the *L. albus* map of Croxford et al. (2008) and found that among the 36 marker loci shared by both maps, there were four pairs of syntenic loci and one triplet (Table 9.4). There were 14 other non-syntenic pairs of shared loci (data not presented). Taken together, these few observations imply a significant amount of chromosomal rearrangement between the genomes of *L. angustifolius* and *L. albus*. Figure 9.4 gives a tantalizing glimpse into possible chromosome rearrangements that distinguish linkage group LG03 of *L. angustifolius* (Nelson et al. 2006) and linkage groups LG1 and LG3 of *L. albus* (Croxford et al. 2008). Too few shared marker loci are available to make strong conclusions, but the available data hint at possible chromosome fission/fusion and inversion events that distinguish chromosomes of these two lupin species.

Table 9.3 Examples of synteny observed between the genome linkage maps of *L. angustifolius* (Nelson et al. 2006) and *L. albus* (Phan et al. 2007)

Syntenic markers	*L. angustifolius* linkage group	*L. albus* linkage group
Lup160, Lup273	LG01[a]	LG16
CPCB2, Lup056	LG02	LG24
Lup003, Lup181	LG08	LG1
Lup088, Lup241	LG11	LG4

[a]Lup160 was located on the minor linkage group “Triplet-1” in Nelson et al. (2006) but was subsequently joined to LG01 (unpublished data)

9.5 Lupin Genome Structure

9.5.1 BAC Libraries

Physical genome mapping, positional gene cloning, and sequencing can be greatly facilitated by the availability of DNA libraries with large inserts. Bacterial artificial chromosomes (BACs) have become the standard vectors for cloning large DNA fragments of plant genomes. Due to the large insert size (10^5 bp), the number of BAC clones needed to cover the genome is relatively low (10^4–10^5 clones). Thus, it is possible to store the clones individually and create ordered libraries representing whole genome, which may be used for the selection of specific clones.

The first nuclear genome BAC library of *L. angustifolius* was constructed in a collaborative project

Table 9.4 Examples of synteny observed between the genome linkage maps of *L. angustifolius* (Nelson et al. 2006) and *L. albus* (Croxford et al. 2008)

Syntenic markers	*L. angustifolius* linkage group	*L. albus* linkage group
Lup130, UWA099	LG01	LG5
Lup275, UWA278	LG03	LG1
Lup019, UWA211	LG03	LG3
Lup060, Lup204	LG10	LG19
LSSR14, Lup063, Lup222	LG19	LG9

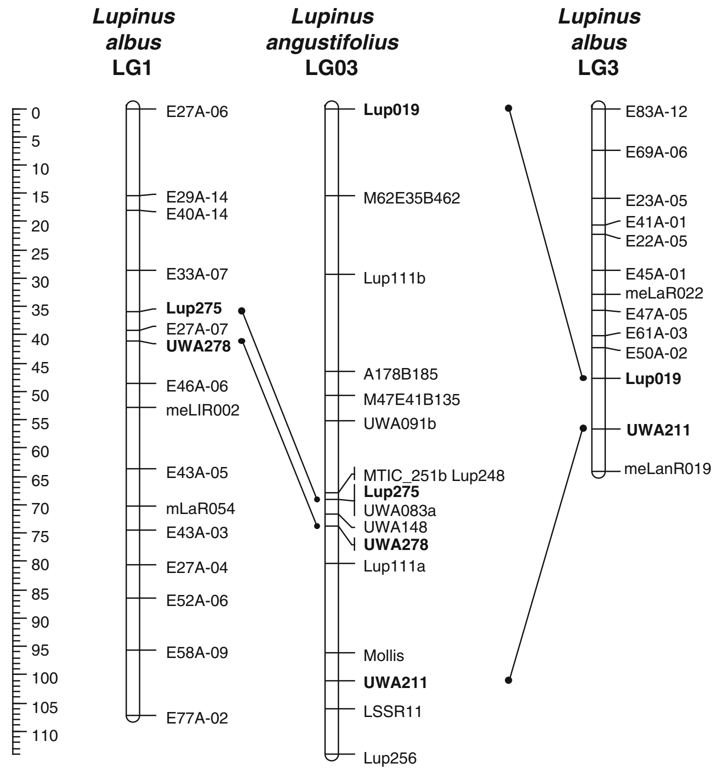


Fig. 9.4 Comparative mapping between *Lupinus angustifolius* and *L. albus* genomes. Four gene-based markers that mapped to *L. angustifolius* linkage group LG03 (Nelson et al. 2006) also mapped to *L. albus* linkage groups LG1 and LG3 (Croxford et al. 2008) are shown in larger font and connected by lines between the linkage groups

between the Institute of Plant Genetics Polish Academy of Sciences, Poznań (Poland) and the Institute of Experimental Botany, Czech Academy of Sciences, Olomouc (Czech Republic) (Kasprzak et al. 2006). The *L. angustifolius* species was chosen as a target because of the relatively low chromosome number ($2n = 40$), moderate genome size of 1.89 pg (2C) (Naganowska et al. 2003a, b), its economic importance, and wide range of cultivation. The library was developed from cultivar Sonet, which is a very early maturing variety that is resistant to viruses, Fusarium disease, and plant lodging. Its genotype contains some domestication genes such as: *iuc* (low-alkaloid content), *ta* and *le* (non-shattering pods), *Deter* (determinate growth habit), *Ku* (early flowering and thermoneutrality), and *moll* (soft seed coat).

One of the crucial problems at initial step of BAC library construction is obtaining high molecular-weight DNA free of contamination with organellar DNA. The improved protocol using flow sorting for the purification of nuclei (Šimková et al. 2003) was used in the lupin library construction. That resulted not only in almost complete absence of organellar DNA (lower than 0.1%) but also in a very high molecular weight of isolated DNA.

The library consists of a total number of 55,296 clones. The key quality parameters of the library are comparable to other plant BAC libraries: the average insert size is 100 kb, representing approximately six haploid genome equivalents and the probability of recovering any lupin DNA sequence from the library predicted to be 99.7%. Therefore, this library is suitable for a wide range of genomics applications. The *L. angustifolius* Sonet genomic BAC library described here is freely accessible for research collaboration with lupin geneticists and breeders. Its availability should stimulate development of physical contig map, positional gene cloning, and further analysis of genome structure in lupin.

The plant biotechnology group at CSIRO (Perth, Australia) in collaboration with Australian and overseas partners constructed a BAC library of *L. angustifolius* cultivar Tanjil (Foley et al. 2008). The library represents 11–12 haploid genome equivalents and is an excellent complement to the Sonet library described above because it has been developed by

using different restriction enzyme (*Bam*HI instead of *Hind*III) and a different genotype. The library was constructed for extending the investigation of lupin resistance genes and seed storage proteins in the perspective of comparative studies of legume crops.

One of the attractive uses for the BAC library is the identification and cloning of genes coding agriculturally important traits, e.g., disease resistance. To address this question, high-density colony arrays can be screened using candidate genes (based on function, generally from a model genome species) or linked markers as probes (Książkiewicz et al. 2008). This will yield a set of BAC clones containing sequence homologous to the probe. The fingerprints of selected clones after digestion with restriction enzymes make possible the construction of contigs in genome region of interest. A number of strategies can then be employed to identify potential candidate genes for the trait of interest in those BACs. The Sonet BAC library is also being used to integrate the genetic and physical maps of *L. angustifolius* and to study the organization of this genome. Molecular cytogenetics can contribute to genome mapping through the assignment of genetic linkage groups to chromosomes (Kaczmarek et al. 2009). Localization of BAC clones using fluorescence in situ hybridization (BAC-FISH) was shown an effective approach to physically map specific DNA sequences and develop chromosome-specific cytogenetic markers (Naganowska and Leśniewska 2008).

9.5.2 cDNA Libraries

cDNA libraries, beside the genome libraries described above, are valuable tools for rapid searching and isolation of specific genes, but first of all, thanks to intensive development of sequencing technology, they are valuable tools for analysis of the whole gene pool, which undergoes expression in different part of plant or tissue and in different stage of growth and development. The series of λgt and λZAP vectors are commonly used for the construction of cDNA libraries. The quality and representativeness of isolated and purified mRNA has a strong influence on quality of the library.

Lupin cDNA libraries have been developed from RNA isolated from different plant tissues, such as mature lupin leaves (Regalado et al. 2000), roots (Attucci et al. 1995; Wasaki et al. 1997; Uhde-Stone et al. 2001; Tian et al. 2009) of *L. albus*, root nodules of *L. angustifolius* (Reynolds et al. 1992; Winefield et al. 1994), and root nodules (Konieczny et al. 1987; Deckert et al. 1997; Narożna et al. 2004) and hypocotyls (Nuc et al. 1997, 2001) of *L. luteus* or cell suspension of *L. polyphyllus* (Perrey et al. 1990). These libraries have become very useful tools for identifying and sequencing new genes, characterization of their expression in different plant organs, and changes in expression of genes under influence of biotic and abiotic stresses. A total of 9,921 ESTs, mainly from the *L. albus* root libraries (Uhde-Stone et al. 2003; Tian et al. 2009), are available in databases at the time of writing (April 2010). In comparison 416 ESTs developed from *L. luteus*, hypocotyl and root libraries were published. *L. angustifolius* genome is represented by 388 published ESTs, mainly from developing seed and nodulated root libraries (Nelson et al. 2006). Foley et al. (2008) reported two cDNA libraries from developing *L. angustifolius* seed and sequenced 1,000 ESTs, but sequences are not yet publicly available. Additionally, the cDNA sequences have been the key to the development of gene-based markers for mapping studies in lupin (Nelson et al. 2006; Phan et al. 2007; Croxford et al. 2008).

9.6 Lupin Domestication and Breeding

9.6.1 History of Domestication and Early Cultivation

Archeological evidence has established that at least two of the grain legumes, pea and lentil, played a role with wheat in settled agriculture in the earliest Neolithic sites of the Middle East during the period 7500–7000 BC. Cultivation of lupins, however, is believed to date from about 2000–1000 BC or earlier in the Mediterranean basin and the central Andean region of South America (Smartt and Hymowitz 1985).

It is generally accepted that in the Old World, Egypt was the country of origin for the agricultural use of lupins (Gladstones 1974). However, there are divergent opinions suggesting that cultivated varieties

could only have been introduced into this area after 300 BC. The earliest historical data on the cultivation of lupins comes from the Mediterranean region of Greek and Roman cultures and were extensively described in German literature (Hondelmann 1984). The species *L. albus* was predominant during that time among cultivated legume crops and it was favored for green manuring and seed harvest due to its ability to grow on poor soils. Seeds were used both for animal and human consumption. Because of their bitterness, seeds were boiled and then leached for a long time to remove most of the water-soluble alkaloids. Some accounts indicate that *L. albus*, after the fall of Rome, was cultivated as a minor subsistence crop throughout the Mediterranean area, around the Black Sea and the Nile Valley up to Ethiopia.

The parallel history of *L. mutabilis* cultivation can be traced in the Andean highlands of the New World, but it is less thoroughly documented. The first evidence of lupin cultivation in South America is linked to the Chavinoid culture from 1000 BC (Gross 1986). Further data comes from Tihuanacoid and then from the Inca civilization, which gave rise to the South American cultivation system and introduced lupin as a regular part of the crop rotation system. Mountain Indians debittered lupin seeds by soaking them for several weeks in bags in mountain streams or steeped them in containers, and the leachate was used as an insecticide (Brücher 1968). The invasion of Spanish conquistadors in the sixteenth century resulted in the decline of the Andean agriculture and pushed lupin cultivation to a few marginal, very high-altitude environments. Interest in the New World lupins has increased again in the last decades.

It seems probable that there were attempts to domesticate some other lupin species, but these were not successful. The human settlement on areas with fertile soil held no advantage for lupins, while their long-term adaptation to the wild environment developed such characteristics as high alkaloid content, pod shattering, and hard seeds.

The beginning of bitter lupin cultivation in northern Europe can be quite precisely determined at the year 1781 when King Frederick II of Prussia officially ordered *L. albus* to be introduced as a crop improving the poor soils of northern Germany (Hondelmann 1984). The efforts of King Frederick II and others largely failed because the available genotypes matured too late in the short summer season. In mid-1800s, *L. luteus* was introduced in that area with more success, and within a short time, it became an essential part of agriculture right across the acidic, sandy soils of Baltic coastal areas to be used for forage or green manuring and for seed production. The third lupin species – *L. angustifolius* – was also established in Europe in the early nineteenth century, first in southwestern France for cattle fodder, in Germany for green manuring, and then in England for sheep folding and improvement of sandy soils (Oldershaw 1925). Its basic advantage in the climate of northern Europe was frost tolerance. The cultivation of bitter *L. luteus* and *L. angustifolius* extended gradually eastward into the acidic soils of Poland, Belarus, Russia, and Ukraine, whereas *L. albus* cultivation remained limited to the warmer climate and more fertile soils of eastern central Europe. Generally, cultivation of bitter lupins decreased significantly at the turn of the nineteenth and twentieth centuries as a result of economic changes, production of cheap nitrogen fertilizers, rising land value, and preference for more intensive agricultural production (Hackbarth and Troll 1956; Gladstones 1998).

The interest in lupin cultivation, especially *L. luteus* and *L. angustifolius*, increased significantly after World War I. New bitter varieties with advanced maturity and soft seeds became popular not only in Germany but also southeastern USA, the Cape Province of South Africa and southern New Zealand. However, lupins still lacked some attributes significant for crop plants. Only *L. albus* and *L. mutabilis* had non-shattering pods at ripeness and only in these species, and in *L. angustifolius*, the trait of soft-seededness had been attained. But the most essential obstacle in the acceptance of lupins as crops was their high alkaloid content, precluding their direct use as food and feed except after inconvenient processing.

9.6.2 Modern Lupin Breeding

The history of lupin breeding can be found in Gladstones (1970), Hondelmann (1984), and Cowling et al. (1998b) with a more specific discussion of Australian lupin breeding in Gladstones (1994) and Clements et al. (2005a) and German work in Hondelmann (2000) and Brummund (2000). The identification of natural mutants of lupins with low

alkaloids by Reihold von Sengbusch and subsequently by others marked the beginning of modern lupin breeding. These simply inherited genes for reduction of alkaloid initiated the first steps in the transformation from a wild plant to a domesticated crop with good biological and seed yields, providing excellent nitrogen supply in rotation on infertile soils.

Early breeding methods mainly involved plant selection following simple hybridization between natural or induced mutants with bitter varieties and landraces. After this initial period of combining simply inherited major traits from natural mutants, came selection through recombination breeding for more complex traits, such as adaptation and yield and for the successive changes in traits, such as flowering time while maintaining protein and low alkaloid levels. Mutation breeding was also used in countries such as Poland and Russia to introduce traits such as restricted branching and recently to develop herbicide tolerance mutants (Si et al. 2006). The four major lupin crop species each have relative differences, although often overlapping plant adaptation and seed quality characteristics (Table 9.5) and breeders have exploited these specificities while enhancing or broadening characteristics within the genetic limits of the intraspecific germplasm base.

9.6.2.1 White Lupin: *Lupinus albus*

L. albus is recognized for its generally wide adaptation and good seed quality. Seed protein content in varies from 33 to 47% and oil content from 6 to 13% depending on genotype and environment (Huyghe 1997; Petterson et al. 1997). Although predominantly self-pollinating, outcrossing rates of 8–10% were reported (Green et al. 1980) in *L. albus*. Breeding of *L. albus* has been conducted typically through pedigree selection of pure lines. The gene *pauper* for low alkaloid content, selected in the early 1930s in Germany, is now most exclusively used in breeding programs (Huyghe 1997), although several other genes are available such as *exiguus* and *nutricius* (Gladstones 1970; Lin et al. 2009). Following the combining of domestication traits such as and soft and white seeds and reduced pod-shattering, which were already each available from ancient times, with that of reduced alkaloids, there was a focus on increased yield and the reduction of flowering time and excessive indeterminate branching. The new low alkaloid cultivars were found to have lower yields than comparable bitter lines (Cowling et al. 1998b; Brummund 2000), and further breeding was required to compensate. The discovery of thermoneutral genotypes (e.g., cv. Neutra 1959) provided greatly reduced time to maturity and tolerance to delayed sowing in spring (Brummund 2000). Reduction of plant height in the so-called "short" types by German breeders in cultivars, such as cvs. Ultra (1950) and Hansa (1954), also facilitated maturity before autumn rains. Breeding objectives from these times forward, which was carried out mainly by European scientists depended on whether the crop was autumn/winter or spring sown. The areas for winter and spring sowing have been broadly outlined for the Mediterranean basin by Cubero and López-Bellido (1986) based on temperature isotherms. Święcicki (1986b) described *L. albus* ideotypes suited to these differing European growing seasons based on the available major genes for flowering time and growth habit (*brev* – early flowering, short growth; *Flor* – early flowering; *con* – shortens first order branches; *Fest* – reduces flowering on higher order branches). The genotypic combinations were: (1) a spring dwarf type (*brev Flor con Fest*) for northern France, southern UK, and middle Poland; (2) a typical spring type (*Brev flor con Fest*) for mid to southern France, Hungary, middle Ukraine, northwestern USA and high rainfall maritime/Mediterranean climates; (3) a late spring type for Mediterranean countries with maritime climates (*Brev flor Con fest*); (4) winter types (*Brev flor Con fest*) for Egypt, Azores, and Canary Islands. Another gene named *ep1* for the "self-completing" character, which restricts branching (determinacy) to the first order, was combined with a spring and spring dwarf backgrounds. Determinacy in white lupin is under monogenetic and recessive inheritance (Julier and Huyghe 1993) and restricts branch orders down to one or two levels and provides earlier maturation in cooler climates (Milford et al. 1993). Winter forms were developed further to incorporate tolerance to low temperature and disease resistance, especially brown leaf spot (*Pleiochaeta setosa*) for which recessive resistance from Italy was identified by M. Lenoble in France (Święcicki 1986b).

Substantial further development of *L. albus* plant architecture from its classically indeterminate growth habit for various European growing conditions has occurred in the past 15 years (Julier and Huyghe 1993;

Table 9.5 General characteristics of the major crop lupin species

	L. albus	*L. luteus*	*L. angustifolius*	*L. mutabilis*
Growing season climate	Cool to moderately warm, some frost tolerance	Mild temperatures, frost susceptible	Cool to mild temperatures, moderately tolerant to frost	Narrow temperature range, very frost susceptible
Soil adaptation	Mildly acid to mildly calcareous loamy sands and loams, very intolerant of waterlogging, cluster roots give efficient P uptake, low cadmium accumulation, manganese accumulator	Strongly to mildly acid sands and sandy loams, some waterlogging tolerance, aluminum tolerant, sensitive to alkaline soils, cadmium accumulator, more effective uptake of P and Zn than *L. angustifolius* (tertiary/secondary clumped lateral roots), high-N-fixing efficiency	Moderately acid to neutral sands and sandy loams, intolerant of waterlogging, low cadmium accumulation	Mildly acid to neutral loamy sands and loams, tolerant of waterlogging, low cadmium accumulation, low P requirement, high internal P-use efficiency
General soil fertility requirement	Moderate	Low	Low to moderate	Moderate
Water requirement	Moderate	Low to moderate	Low	Moderate
Fungal diseases	Very susceptible to anthracnose but some resistance *anr-2*. Susceptible to fusarium, rust, botrytis. Resistance to phomopsis generally although susceptibility reported in S. Africa.	Susceptible to anthracnose Single gene resistances available for fusarium. Resistant to Pleiochaeta root rot and good sources of resistance to brown spot (polygenic). Moderate resistance to phomopsis, Eradu patch resistance	Susceptible to anthracnose, moderate resistance available (*AnR-1* and other genes). Susceptible to fusarium. Susceptible to Pleiochaeta root rot and brown spot but polygenic resistance available. Susceptible to phomopsis but resistance available (*Phr1*, *Phr2*)	Susceptible to anthracnose but less so than *L. albus*. Resistance available to fusarium. Very susceptible to Pleiochaeta root rot and brown spot but some genetic variation. Relatively resistant to phomopsis.
Virus diseases	CMV: tolerant. BYMV: moderate problem and seed borne in East and central Europe, and USA; not seed-borne in Australia. A non-necrotic strain causing concern in Australia.	CMV: susceptible. Serious problem in East and central Europe – carried over through lupin seed. Resistance to seed transmission occurs - single gene resistance *Ncm-1*. BYMV: serious problem in East and central Europe, and in USA – carried over through lupin seed. Resistance to seed transmission occurs. Partial resistance to BYMV infection used in breeding in Europe. Very low alkaloid lines susceptible to aphids	CMV: highly susceptible. Serious problem, seed-borne, partial resistance to seed transmission used in breeding in W. Australia. BYMV: serious problem, high susceptibility, not seed-borne, partial resistance to infection by aphids occurs. Two strains, necrotic and non-necrotic. Aphid resistance	CMV: susceptible. Moderate problem, seed transmission not recorded, resistance found in one line. BYMV: highly susceptible.
Herbicide tolerances	Moderate tolerance to simazine and diflufenican, susceptible to metribuzin, tolerant to grass selective herbicides	Moderate tolerance to simazine and diflufenican, susceptible to metribuzin, tolerant to grass selective herbicides	Tolerant to simazine and diflufenican, some cultivars tolerant to metribuzin, tolerant to grass selective herbicides	Moderate tolerance to simazine, susceptible to metribuzin, tolerant to grass selective herbicides

(*continued*)

Table 9.5 (continued)

	L. albus	*L. luteus*	*L. angustifolius*	*L. mutabilis*
Protein (% in seed)	36.1	38.3, higher S-amino acids	32.2, γ conglutin storage protein	42.0
Oil (% in seed)	9.1	5.6	5.8	18.0
Lysine	1.58	2.07	1.46	2.56
Cysteine + cystine[a]	2.3	3.2	2.0	[b]
Methionine	0.24	0.27	0.20	0.31
Seed coat (% of seed)	18	25	24	13
Pod wall (% of whole pod)	28	42	32	45
Other traits	Specific antioxidants (lutein and others), higher isoflavone levels (genistein)			Very thin seed coat, low NSPs
Main alkaloids	Lupanine, 13-hydroxylupanine, albine,[c] angustifoline, multiflorine, isolupanine	Lupinine, sparteine, gramine[c]	Lupanine, 13-hydroxylupanine, angustifoline, isolupanine	Lupanine, 13-hydroxylupanine, sparteine

From Gladstones (1970), Hove et al. (1978), Culvenor and Petterson (1986), Röemer and Jahn-Deesbach (1988), Jones and McLean (1989), Wink et al. (1995), Petterson et al. (1997), Caligari et al. (2000); Cheng and Jones (2000), Jones (2001), Cheng et al. (2002), Jones et al. (2003), Clements et al. (2002, 2004), Sipsas et al. (2004) and Sweetingham et al. unpublished

[a]From Sipsas et al. (2004) using method of Barkhold and Jensen (1989)

[b]Not available

[c]Variously present in some lines

Milford et al. 1993; Huyghe 1997, 1998). This was achieved through the use of determinacy and dwarfism. Determinate forms of *L. albus* were found to have reduced intrainflorescence variation in seed size and higher harvest index than indeterminate types. A more uniform and, in some cases, larger seed size is desirable for processing in some markets. While grain yield in indeterminate genotypes in maritime temperate environments is not correlated with biomass, it is in restricted branching genotypes. Peel and Galwey (1999) suggested that stable yields for these environments in autumn-sown *L. albus* could be achieved by combining a low vernalization requirement with a long juvenility period despite the two traits being positively correlated.

L. albus is relatively widely adapted with respect to soil types (Gladstones 1984). This species produces numerous, well-defined proteoid roots, which are induced in response to low phosphorus and iron in the soil (Clements et al. 1996; Hocking and Jeffery 2004). Proteoid roots enable *L. albus* to take up almost five times more phosphorus per unit root length than soybean, which does not form them (Watt and Evans 2003). Germplasm with good tolerance to the abiotic stress induced by calcareous soils has been identified from Egypt (Christiansen et al. 1999) and from other regions such as the Bari region, Italy (Huyghe 1997). Some *L. albus* material from Egypt was found to have tolerance to limed soil at levels found in the tolerant species *L. pilosus* (Kerley et al. 2002).

Variation for cold and frost tolerance (Huyghe 1997) have been used for breeding and sources of cold resistance are likely to be found in germplasm of *L. albus* var. *albus* and var. *graecus* from Italy, Spain, Turkey, and the Greece. Despite some evidence for genetic variation for physiological tolerance to drought (Rodrigues et al. 1995), the main avenue of progress in *L. albus* has been achieved through developing the correct phenology, i.e., altering flowering time and plant architecture. Little remobilization of assimilates stored in the stem to seeds occurs in *L. albus* (Withers and Forde 1979). Development of deeper roots while maintaining yield may be another characteristic worth exploring in this species. A more extensive root system and higher leaf area and stomatal conductance gave better drought tolerance in cv. Acores (Rodrigues et al. 1995).

Current breeding in white lupin is especially focused on improving anthracnose resistance (*Colletotrichum gloeosporioides*), a disease which limits yields in most countries where it is currently grown (Adhikari et al. 2006b). The other major fungal diseases affecting *L. albus* are Pleiochaeta root rot and brown leaf spot (*P. setosa*), rust (*Uromyces lupinicolus*), Fusarium wilt (*Fusarium oxysporum* f. sp. *lupini*), and gray mold (*Botrytis cinerea*). Anthracnose was a problem in Central and South America but emerged more widely in the past 15 years in Europe and other countries. Apart from recessive resistance from Italian germplasm reported by Święcicki (1986b), resistance to Pleiochaeta root rot has been reported in germplasm from Crete and the Azores (Sweetingham and Yang 1998). Reference to some levels of tolerance to anthracnose occurs (Talhinhas et al. 2000; Clements et al. 2005a; Adhikari et al. 2006b). Collaborative screening work has identified some promising resistances (Sweetingham et al. 2006b), and there is potential to find further sources of resistance to anthracnose in germplasm (e.g., from the Azores and Iberian Peninsula). *L. albus* appears to be resistant to phomopsis, compared to *L. angustifolius*, although there is marked strain specificity (Shankar et al. 1999). While *L. albus* is inherently resistant to CMV (Jones and Latham 1996), it is susceptible to bean yellow mosaic virus (BYMV), being a moderate problem in East and central Europe and USA. The strains that are seed-borne are not present in Australia. However, a non-necrotic strain has been identified in Australia, and this is causing some concern (Cheng and Jones 2000). Sowing seed with minimal BYMV is the most important control strategy.

9.6.2.2 Narrow-leafed Lupin: *Lupinus angustifoloius*

L. angustifolius is regarded as a self-pollinated species, with automatic self-pollination occurring usually before the petals open. Outcrossing rates have been reported to range from practically 0 (Wallace et al. 1954) to 2% (Dracup and Thomson 2000) with a report of up to 12% (Forbes et al. 1971). Narrow-leafed lupin breeding began in Germany, Poland, and also in Russia, with the discovery of low alkaloid natural mutants (Gladstones 1970, 1998; Święcicki and Święcicki 1995; Kurlovich 2002a), and was followed by the intensive work in Australia, beginning in the late 1950s, and some parallel and collaborative work in USA (Gladstones 1994). The crop has played a very

important role in Australia since the early 1980s in rotation, particularly with cereals as a feed for sheep, pigs, and poultry. It is also grown currently on a commercial scale in Poland, Byelorussia, Baltic countries, Russia, South Africa, and Chile, its popularity due to reliable maturation in summer-grown crops, reasonable disease resistance, good yields, and good adaptation to a range of environments.

Lines of *L. angustifolius* evaluated in the 1950s in Australia were initially very late flowering and not productive in the environments in which they were first tested. The crop improvement program based in Perth by J. S. Gladstones focused on *L. angustifolius*. Genes that were found as naturally occurring mutants in cv. New Zealand Blue for non-shattering pods (*le* and *ta*) and white flowers and seeds (*leuc*) were combined with the naturally occurring mutant gene for early flowering and thermoneutrality (*Ku*) along with low alkaloid (*iuc* from cv. Borre) and the permeable seed gene *moll*. A series of cultivars beginning with cv. Uniwhite in 1967 was the beginning of the highly successful history of narrow-leafed lupin in Australia (Gladstones 1994). The program relied upon white flowers and seed as markers and the strict removal of bitter plants to ensure quality and purity in cultivars. Similar to the European experience with yellow lupins, a negative effect of domestication genes on yield and other characters occurred in *L. angustifolius*. The *iuc* gene for low alkaloid and, to some extent, the *ta* gene for non-shattering had negative pleiotropic effects on the severity of the split-seed disorder caused by manganese deficiency on infertile sandy soils (Walton and Francis 1975). Isogenic lines of earlier Australian *L. angustifolius* breeding material with the *iuc* gene were 30% lower yielding than bitter lines (Oram 1983). It is not surprising with these kinds of results for *L. angustifolius* and with the general observation that low alkaloid lupin crop genotypes have both greater pest susceptibility and possibly lower inherent yield potential that the concept of the "bitter-sweet" plant ideotype has arisen. The "bitter-sweet" concept proposes the generation of a plant with high alkaloids in vegetative tissues but which does not translocate alkaloids to or produce them within seeds possibly through the manipulation of alkaloid transporters or seed-specific promoters (Wink 1991, 1994). Although the gene *le* for reduced pod shattering through modification of the pod endocarp (Gladstones 1967) had some negative effects on plant growth, the gene *ta* for reduced shattering had positive effects on plant growth and yield. Breeding over the past 20 years has improved yield to compensate for this apparent physiological deleterious effect of the gene for lower alkaloid content, which is in addition to the affect of greater general susceptibility to insects and in some cases diseases.

Following the initial phase of domestication, the next phases of breeding *L. angustifolius* in Australia were the incorporation of disease resistance genes from wild germplasm and selection for yield improvement through both pedigree selection and recurrent selection breeding methods (Gladstones 1994; Cowling and Gladstones 2000). Use of F_1s in crosses back to elite breeding lines was also common in the Australian program since the 1980s. Traits incorporated included gray leaf spot (gl_1) and anthracnose (*An*) resistance from USA cv. Rancher, phompsis resistance from Spanish and Moroccan wild types, and brown leaf spot and Pleiochaeta root rot resistance from Israeli and Italian wild types (Cowling and Gladstones 2000). With the outbreak of anthracnose in 1996 in Australia, intensive efforts were made to screen existing cultivars and germplasm for resistance both within Australia and offshore (Cowling et al. 2000). The pre-emptive intermediate resistance possibly originating from American breeders (*An* gene, Gladstones 1970) that was available in cvv. Illyarrie and Yandee and stronger resistance from cvv. Tanjil and Wonga (*Anr1* gene, Yang et al. 2004) has indicated that the disease is a lower threat in *L. angustifolius*. Another separate moderate resistance exists in cvv. Kalya and Mandelup (Yang and Buirchell unpublished).

The races of the Fusarium wilt pathogen in Germany, Poland, Ukraine, Belarus, and Russia vary in their specificity between *L. angustifolius*, *L. albus*, and *L. luteus* (Lamberts 1955). Local breeding programs have bred for resistance in each region, but resistant cultivars do not always show resistance elsewhere. *P. setosa* causes brown leaf spot in *L. angustifolius* but can be controlled by minimum tillage, stubble retention, and fungicide seed dressings. Recurrent selection in Western Australia produced cv. Myallie with improved resistance (reduced defoliation), which is polygenic in nature (Cowling et al. 1997). The mycotoxin of Phomopsis (*Diaporthe toxica*) has caused lupinosis in sheep in the past, usually without major effects on seed yield (Cowling and Wood 1989; Williamson et al. 1994). A dominant (*Phr1*) resistance gene originating from the breeding line 75A258 with

a Moroccan wild type parent and an incompletely dominant gene (*Phr2*) for resistance in cv. Merrit have been developed in Australia (Shankar et al. 2002). Two DNA markers are available for *Phr1* (Yang et al. 2001).

CMV is a serious problem in *L. angustifolius*. The species is highly susceptible, particularly in late flowering cultivars. The virus is seed-borne with partial resistance to seed transmission used in breeding in Western Australia and present in some local cultivars, which is probably polygenically controlled and not related to plant alkaloid content (Jones and Cowling 1995; Jones 2001). BYMV is also a serious viral disease in *L. angustifolius*, which has high plant susceptibility with seed transmission depending on isolate. Western Australian isolates are not seed-borne but seed transmission occurs in other countries. Necrotic and non-necrotic strains of BYMV occur, the necrotic reaction being a form of systemic hypersensitivity.

Yield improvements in *L. angustifolius* have been through a combination of phenological improvements and disease resistance. In wild and landrace germplasm lacking any early flowering genes, time to flowering appears to be influenced primarily by vernalization with only minor influence of photoperiod (Rahman and Gladstones 1974). Landers (1995) showed that this vernalization response can range from an absolute (essential for flowering) to a reduced response (vernalization not essential as provided by the *efl* gene) to no response in genotypes carrying the *Ku* gene for early flowering. Further research on a wider range of breeding lines and germplasm could reveal useful variation to match biomass production prior to flowering to specific environments. Although high yielding Australian cultivars carry the *Ku* gene and are of indeterminate branching architecture, the restricted branching trait has proven useful in European programs. Yield increases of approximately 2–3% per year have been achieved from the first cultivar release in Western Australia in 1967 to the present (Clements et al. 2005a; Berger et al. 2008b). There have been progressive increases in harvest index from approximately 0.24 to approximately 0.34 in the latest cultivars (Tapscott et al. 1994; Clements et al. 2005a). Comparable figures of both harvest index and water use efficiency show that lupins still fall below that of some other legumes, such as *Vicia faba* and *Pisum sativum* (Siddique et al. 2001). The changes in harvest index in historical Australian cultivars has been associated with improved pod set and yield at higher plant densities (Tapscott et al. 1994), decreases in plant height, and more recent reductions in upper primary branch length since cultivars such as cv. Danja (1986) with long lateral branches. Drought tolerance is achieved through earlier flowering and, in more recent cultivars, rapid pod set and seed growth (Palta et al. 2003, 2004). G $\times$ E analysis of narrow-leafed lupin historical cultivars indicated little specific adaptation to environments, and this suggests the need for new adaptation genes that could increase yields in specific environments (Berger et al. 2008b).

9.6.2.3 Yellow Lupin: *Lupinus luteus*

Outcrossing rates indicate that *L. luteus* is both self-compatible and possesses the ability to outcross to moderate levels of approximately 8% (Adhikari et al. 2006a) but with some reports for mingled plants of up to 40% (Wallace et al. 1954). The main centers of yellow lupin breeding have been Germany, Poland, Belorussia, and Russia. Low alkaloid genes reduced the levels of the major alkaloids of yellow lupin, sparteine, and lupinine from their levels of 1–4% in seed and 0.2–0.5% in shoot dry matter. Levels of from 0.08 to 0.1% as in cv. Teo to as low as 0.02% as in cv. Wodjil have been achieved using the original natural mutant genes for low alkaloid (Cowling and Gladstones 2000). Other minor alkaloids, such as gramine, which were only discovered after the development of improved methods of alkaloid analysis, occurred in all *L. luteus* wild and landrace germplasm. This alkaloid was found to have a negative influence on fodder palatability. A progressive decline in the presence of gramine apparently occurred in breeding material in Poland, although higher amounts have still been found in some lines. For example, in cv. Teo, approximately 90% of the alkaloids are gramine (Święcicki and Święcicki 2000). Three low alkaloid genes were identified in yellow lupin (*amoenus*, *liber* and *dulcis*), one of which was associated with poor vigor (*liber*). The gene *dulcis*, developed by von Sengbusch, appears to have been used more frequently in breeding programs (Gladstones 1970; Pate et al. 1985). Reduced pod shattering was provided through the gene *invulnerabilis*, which provided large yield increases in European crops of yellow lupins. In Mediterranean environments with hot conditions at the end of the

season, pod shattering still occurs, and there is a need for additional or alternative genes to reduce this.

After initial domestication by combining a number of recessive alleles (including *w* for permeable seeds), further improvements in yellow lupin were achieved by creating more rapidly growing and earlier flowering genotypes with higher yield, both for green and dry fodder and for seed (Święcicki and Święcicki 2000). This was achieved through the identification of genes such as *crescens celer* (*cres*^{cel}) in cv. Weiko III and *crescens altus* (*cres*^{alt}) in cv. Alteria, both genes giving a lighter green foliage color. Another gene *promptus* (*prompt*) accelerates growth but does not affect leaf color (e.g., cv. Expires). Additionally, the gene *Rapidus* (*Rp*), from the Netherlands, gives rapid early growth and had a pleiotropic effect on dark green leaf color and gave a stronger main stem (cvv. Palvo, Juno, Teo). Lines carrying the genes *cres* and *alt* together were found to have reduced root growth and therefore yield reductions under certain environments (Brummund 2000). The saturation of breeding material with the recessive alleles in the period from 1940 to the 1970s in several cultivars such as Refusanova resulted in lower yields relative to bitter types or those carrying few recessive genes. Cultivars after that period, which contained fewer of these genes, were found to recapture higher yield levels (Brummund 2000; Święcicki and Święcicki 2000). One of the genes dispensed with was the *alb* gene for white seed coat, which was no longer seen as being necessary in German programs.

Plant architecture is typically characterized by an early rosette stage followed by indeterminate branching. The gene *Therm* provided thermoneutrality and therefore removed vernalization requirement, resulting in earlier flowering even when crops were sown in late spring (Święcicki and Święcicki 2000). The gene also provided escape from viral infection. A gene for restricted branching, *rb*, was discovered in Hungary and introduced into a range of cultivars such as Manru, Borselfa, Radames, Markiz, and Legat. Genotypes with this gene were referred to as "self-completing" yellow lupins, and they provided advantages similar to the analogous trait in *L. albus*. Święcicki and Święcicki (2000) found restricted branching cultivars to be highly suited to higher rainfall but shorter growing seasonal environments.

Yellow lupin has some susceptibility to frost, particularly in the earlier growth stages as well as when racemes are exposed to transient frosts during flowering and podding. Interspecific crossing with *L. hispanicus* ssp. *hispanicus* and ssp. *bicolor* could offer resistance to low winter temperatures (to $-15°C$) (Święcicki 1986a), important for inland Spain and for extending the crop into northern Europe. This would depend on whether germplasm could be found that does not merely escape frost through late flowering. Yellow lupins are tolerant of soil aluminum (French et al. 2001) and are best suited to soils from pH 4.5–6.5. While *L. luteus* is more efficient at taking up soil phosphorus, it takes up cadmium, often to above maximum permissible health standard levels (Brennan and Bolland 2003). A possible reason for this efficient uptake of nutrients is the ability of *L. luteus* to produce a large number of third-order lateral roots with a structure not dissimilar to proteiod roots (Brennan and Bolland 2003) and due to the exudation of organic acids at levels similar to *L. albus* under P deficiency (Hocking and Jeffery 2004). *L. luteus* has been found to be relatively waterlogging-tolerant, a trait of some importance in the wetter months of autumn-sown crops in Mediterranean environments (Davies et al. 2000). *L. luteus* accumulates less ABA in roots, relative to other lupin species (Hartung and Turner 1997), and is considered to be drought-susceptible (compared with *L. angustifolius*) in the Mediterranean climates, and the species is better suited to higher rainfall cropping zones (Gladstones 1994). Święcicki and Święcicki (2000) suggest that genotypes with a higher concentration of anthocyanin and chlorophyll (through the gene *oliv* for leaf color) but with short growing periods are more resistant to drought than types having genes for fast growth and with the light green leaf gene *au*.

Very low alkaloid *L. luteus* genotypes, such as cv. Wodjil, can be severely damaged by aphids (Berlandier and Sweetingham 2003). Efforts are underway to determine mechanisms of tolerance and breeding for tolerance by maintaining alkaloid levels at 0.02% or below but by manipulating alkaloid profile (Adhikari et al. 2003; Edwards et al. 2003). CMV is a serious problem in East and central Europe, where it is carried over through lupin seed and causes "lupin browning disease." Resistance to both plant infection and to seed transmission is available. Resistance to seed transmission is not related to alkaloid content or flowering time and is probably polygenically controlled (Sweetingham et al. 1998). A single dominant hypersensitivity gene, *Ncm-1*, has been identified, which is responsible for resistance to CMV in cv. Wodjil and other genotypes

(Jones and Latham 1996). BYMV is a serious viral disease in East and central Europe, where it reduces yield and is often called "lupin narrow leaf virus." It also occurs in the USA. The virus is readily carried over through lupin seed. Partial and quantitative resistance to BYMV has been used in breeding in Europe, particularly through the use of thermoneutral types, which are less likely to become infected (Sweetingham et al. 1998; Święcicki and Święcicki 2000).

Fungal diseases of yellow lupin include anthracnose, Fusarium wilt, and powdery mildew. Cultivars have moderate resistance to phomopsis (Shankar et al. 1999). Fusarium wilt has caused large yield losses in the past in Europe, and out of three races, races 1 and 2 were the most pathogenic on *L. luteus* (Sweetingham et al. 1998). Resistance was first identified in Portuguese germplasm and later found in other populations from Sicily (Święcicki and Święcicki 2000). The gene Fus^{1} was then transferred to cultivars such as Refusanova, Borluta and Bornova (Germany), and Afus and succeeding Polish cultivars. The resistance provided by this gene has been remarkably stable over time (Święcicki and Święcicki 2000). Resistance to powdery mildew was provided first by Portuguese germplasm then by the gene *Er*, which was derived from Spanish germplasm (Lamberts 1955; Gladstones 1970). Crop rotation, fungicide seed dressing, and use of clean seed can provide control, and some promising levels of tolerance have been identified in collaborative screening programs (Sweetingham et al. 2006b). There has been a report of sources of resistance from Portuguese lines (Sweetingham 2000).

Further breeding of *L. luteus* is required for anthracnose resistance, higher harvest index, and yield, accompanied by lodging resistance, aphid tolerance, and reduced pod shattering. Exploration of different combinations of various genes for earliness, vigor, and plant architecture may provide improvements. Reduction of pod wall proportion, which is unusually high in this species, may also provide yield increases (Clements et al. 2002). Seed coat proportion of total seed weight is also relatively high (Clements et al. 2002, 2005b), and some reduction could be achieved through increases in seed size or through identification of thinner seed coat in germplasm or mutation populations. Yield improvements of approximately 30% over cv. Piast (registered in 1987) have been achieved in Poland with a series of cultivars released over the past decade.

9.6.3 Importance as a Crop

The three species that have achieved modern crop status such as *L. angustifolius*, *L. albus*, and *L. luteus* are from the Old World. *L. cosentinii* Guss., *L. pilosus* L., and *L. atlanticus* Gladst. have also been domesticated, although not used widely (Buirchell and Cowling 1992). The New World species, *L. mutabilis*, has been domesticated but has yet to be used on a large scale as a crop plant. Each of these species, however, possesses useful adaptation, plant, and seed quality attributes that makes the genus a valuable resource for farming practice, production, and use in established feed and emerging food and health industries. Lupin's role in modern agricultural systems began in the twentieth century with the domestication of the three major species, particularly through the development of reduced shattering and low alkaloid lines.

While historically lupins have played a major role as an animal feed, subsistence food, and soil improvers, current and future use has entered the arena of health food and functional ingredient as well as its continuing role in animal nutrition and aquaculture feeds. World grain lupin production peaked during the latter part of the 1990s (Table 9.6) largely as a result of the expansion in Australia. Rapid increases over the past few decades have also occurred in Chile and Germany while declines have occurred in Poland, Italy, and South Africa. A large proportion of lupins in Chile are directed towards salmon aquaculture in the south of the country. In 2007 major producing countries were Australia (despite declines since 1996–2000), Germany, Chile, and Poland followed by sustained lower levels in South Africa, Morocco, and France. Reasons for the contraction of lupins in Europe include anthracnose and Fusarium wilt diseases and competition with soybean imports. The former USSR had large areas (more than half a million ha in the 1950s as forage and green manure) of primarily *L. luteus*, but this declined due to the spread of Fusarium wilt. Better resistance has seen a steady increase in regions such as Ukraine and Belarus. *L. angustifolius* cultivation has increased in Europe due to better disease resistance and more reliable maturity at harvest. In the UK, a rapid increase in the use of lupins in lupin/cereal forage mixtures has occurred. In the Andes in South America, *L. mutabilis* continues to be used as a traditional crop, while in Argentina, there is interest in expanding *L. albus*

Table 9.6 World lupin production for major producing countries (average annual production, metric tons)

Country	1961–1970	1971–1980	1981–1990	1991–1995	1996–2000	2001–2005	2006	2007
Australia	1,410	57,550	593,620	1,271,450	1,560,410	984,800	174,000	331,000
Chile	0	2,070	14,390	30,690	33,240	45,370	70,480	51,400
Egypt	7,960	5,820	7,270	6,350	5,230	3,890	2,790	2,900
France	0	0	1,810	10,820	14,820	26,770	16,560	13,000
Germany	14,580	10,800	12,640	1,110	0	98,330	85,000	85,000
Italy	25,530	8,310	4,740	4,560	4,680	4,790	6,000	5,000
Lithuania	0	0	0	670	1,340	2,400	4,800	7,900
Morocco	1,000	1,820	2,690	3,260	11,660	14,000	14,000	14,000
Peru	1,330	2,580	3,510	3,510	7,220	9,540	9,480	8,500
Poland	155,900	71,780	99,860	74,200	31,760	15,090	27,990	28,000
Russia	–	–	–	10,870	15,340	27,810	13,510	9,500
South Africa	42,830	6,600	7,100	6,600	11,470	10,230	14,400	15,000
Spain	6,350	2,090	1,700	4,630	12,010	9,160	6,900	5,200
USSR	449,000	312,500	265,800	116,200	–	–	–	–
Ukraine	–	–	–	–	–	9,730	6,600	6,000
Belarus	–	–	–	–	–	72,400	54,130	46,000
Total (including minor countries)	720,390	489,870	102,310	1,548,630	1,713,340	1,239,460	424,760	632,050

Source: FAOSTAT (2009)

cultivation in rotation with cereals into areas of the Pampas. Despite competition from other crops, there is increased interest in lupins in southern USA for use as a late winter, high protein livestock feed and as a cover crop for cotton (van Santen et al. 2006). The use of genetically modified lupins would suit such applications in the USA in the future. In both South Africa and other countries close to the centers of diversity for lupins, such as Ethiopia and Kenya, there is considerable potential for mixed uses of lupins in the farming system and for food security. Given the increased interest in lupins for food, for plant-derived proteins in expanding livestock and aquaculture markets, and with the need for reduced fertilizer input and nutrient leaching into subsoil, lupins can offer a viable option for world agriculture in the future. Profit margins for lupin production could be increased through more efficient weed control, increased yield and yield stability, and by increasing grain quality.

9.6.4 Molecular Breeding

Molecular markers can be developed to tag genes of agronomic traits of interest and be applied for marker-assisted selection (MAS) in plant breeding in order to increase the efficiency of genetic improvement. Marker development for practical MAS in lupin breeding started in 2001, and the scale of MAS has been continuously increased. Since 2005, about 15,000–25,000 progeny plants from the lupin breeding program in Australia have been screened and selected annually with molecular markers linked to various genes of interest (Yang et al. 2002, 2008; You et al. 2005; Lin et al. 2009).

The first challenge in developing markers applicable to practical MAS is the requirement for the markers to be closely linked to genes of interest and able to be cost-effectively applied to a large number of samples. At present, sequence-specific, simple PCR-based markers best satisfy the requirements as implementable markers for MAS, and these include sequence-tagged microsatellite site (STMS), sequence-characterized amplified region (SCAR), sequence-tagged site (STS), and allele-specific PCR (AS-PCR) markers.

In lupin, the DNA fingerprinting method of microsatellite-anchored fragment length polymorphisms (MFLP) has played a pivotal role in marker development for marker-assisted selection (MAS) (Yang et al. 2001). MFLP is highly efficient in generating candidate markers, and MFLP markers can be converted relatively easily into sequence-specific PCR markers desirable for routine implementation (Yang et al. 2001, 2002, 2004). In *L. angustifolius*, MFLP-derived sequence-specific markers have been developed that are linked to genes for anthracnose resistance (Yang et al. 2004, 2008; You et al. 2005), for phomopsis resistance (Yang et al. 2002),

for early flowering gene *Ku* (Boersma et al. 2007a), for soft-seed gene *moll* (Boersma et al. 2007b), and for non-shattering genes *ta* (Boersma et al. 2009) and *le* (Boersma et al. 2007c). In *L. albus*, MFLP-derived sequence-specific markers include the marker "PauperM1" linked to the low alkaloid gene *pauper* (Lin et al. 2009) and three markers (WANR1, WANR2, and WANR3) linked to QTLs conferring anthracnose resistance (Yang et al. 2010).

The second challenge for a broad implementation of MAS is that the markers need to be applicable to a wide range of crosses in a breeding program (Holland 2004). In practical MAS in lupin breeding, some markers were found, such as the markers linked to the *Ku* gene (Boersma et al. 2007a), which were only applicable to a very limited numbers of crosses. Similar to the cases in other crops, most of the molecular markers developed for MAS are not "perfect" (i.e., not part of the gene itself), and certain genetic distances exist between the markers and the genes of interest. As a result, cultivars showing the desirable markers may not necessarily possess the targeted genes and vice versa ("false positive") (You et al. 2005). Having learnt the lessons, a new strategy has now been adapted in marker development work in lupin, where a number of candidate markers are generated by MFLP first; the obtained candidate markers are then validated on key cultivars and breeding lines; and only the candidate markers with the widest applicability are selected and converted into sequence-specific markers for routine MAS in lupin breeding (Yang et al. 2008).

9.7 Future Prospects, Existing Limitations, and Priorities

The agricultural potential of lupin species is just now being reached. Three Old World species (*L. albus*, *L. angustifolius*, *L. luteus*) and one from New World gene pool (*L. mutabilis*) can be considered as domesticated and accepted in the market. However, the domesticated gene pool of modern lupins is narrow in comparison to other major crop species. In fact, direct crossing with wild populations or landrace types is a good way to expand this gene pool. A consequence of the recent domestication is that domesticated lupins are still close genetically to their wild relatives so that significant improvements can be achieved through crosses with them. The prospects for improving the adaptation of lupin crops to different climatic conditions and demands of modern agricultural systems through new scientific and breeding technologies are still excellent. Several more species, such as *L. cosentinii*, *L. pilosus*, or *L. atlanticus*, have reached different stages of domestication and each is adapted to different environmental conditions. Domestication of new species can contribute to full exploitation the whole ecological potential of the genus and will provide new crops adapted to a wider range of soil and climate conditions (Gladstones 1970).

9.7.1 Germplasm Enhancement

Interspecific crossing within Old World, the New World, and between these two major groups will dramatically increase the genetic base available to breeders. Already, interspecific crossing among the rough-seeded species has led to the full domestication of several base species within that taxonomic clade. Recent progress in developing hybrids between *L. angustifolius* and *L. luteus* will add a new dimension of genetic base broadening for each of those species if introgression of traits can be achieved whilst maintaining productivity. Crossing among the New World species, a large number of which share the same chromosome number, should be relatively easy for large groups of taxa, given the phylogenetic relatedness and sheer number of species from which to choose. The genetic variability produced through hybridization among these species, which occupy broad habitats, should be very valuable in the future when breeding lupins for specific environments.

Further evaluation and collection of germplasm from higher altitudes may offer some source of frost resistance and from low rainfall environments for drought tolerance and water use efficiency. Breeding *L. luteus* to tolerate higher soil pH may be possible through the use of germplasm that collected from higher pH sites. Some accessions held in Australian gene banks have been collected by C. M. Francis in Morocco on soils with a pH ranging from 7 to 8.5. Screening for variation among breeding lines or germplasm for uptake of cadmium for bioremediation of contaminated soils may be worthwhile. Little information is available on the status of *L. luteus* in relation to

drought tolerance, and a collection of *L. luteus* from drier climates may be worthwhile. More detailed studies to determine if there is genetic variation for nutrient use efficiency both within and among species (including the vast genetic resource in the New World) may be worthwhile in efforts to reduce fertilizer use. A better understanding of the genetics of alkaloid profile in *L. luteus* may provide benefits for aphid resistance, a continuing problem encountered by breeders in Australia. Linking collection site, morphological evaluation data, and genetic data should greatly enhance the smarter use of germplasm for breeding programs and will generally ensure a broad genetic base in sustainable lupin crop improvement.

9.7.2 Use in the Agricultural System and in Feed and Food Markets

World requirement for feed grain, boosted by economic growth in China and India, and the use of grains in biofuels is expected to sustain grain prices and demand. The role of lupins as a feed and use in sustainable cereal and canola crop production will ensure a future for lupins. As part of the trend towards the increased use of plant-derived ingredients in formulation of foods such as dairy and meat analogs, lupin has attracted interest worldwide as a potential high protein food ingredient suitable for human consumption (Sipsas 2008; Sweetingham and Kingwell 2008). New information on the health-promoting properties is emerging, which can bring lupin into roles traditionally played by soybean as well as promoting an identity in the market separate from soybean (Sipsas 2004; Johnson et al. 2006; Lee et al. 2006, 2009). This will increase the need for nutritional studies and molecular characterization of lupins to assist breeding for improved seed quality traits. However, care should be taken in introducing lupin products into the human diet, given the potential for allergic reactions similar to peanut (Goggin et al. 2008).

Characteristically high non-starch polysaccharides found in the Old World lupins, particularly *L. angustifolius* and *L. luteus*, are both valuable dietary fibers with cholesterol-lowering properties and negative components that reduce the digestible energy of feed in monogastrics. Better screening methods for this and other seed quality traits such as lutein, β-carotene, and antioxidant compounds will assist the more efficient and rapid development of higher value seed. Sources of genetic variation could be found in germplasm, through wide crossing or by genetic modification. A premium for yellow lupin grain is possible in Australia because of its greater protein, lysine, and sulfur amino acid content (Petterson et al. 1997). A better assessment of available variation in breeding lines and germplasm for protein and sulfur amino acid content in *L. luteus* may be possible with improved analytical methods (Sipsas 2004; Sipsas et al. 2004) and rapid screening techniques such as near infrared spectroscopy (Pazdernik et al. 1997). Potential for selecting higher protein content in *L. angustifolius* and gerrmplasm may be possible based on the data of Gladstones and Crosbie (1979) and Buirchell (2008).

Genetically modified (GM) crops such as in soybean have escalated in use in the USA and Brazil. Genetic modification offers more efficient weed management in modern farming systems. It is likely that the development of GM lupins will progress in the future, initially in crops destined for feed markets, and this will open new research opportunities. The major focus will initially be herbicide resistance; however, seed quality and fungal and insect resistance traits may be additional candidates for GM lupins.

9.7.3 Mapping Populations and Markers

Due to the foresight of lupin genetic researchers and breeders in Australia and the UK, there exist valuable RIL mapping populations for three of the major lupin crop species: *L. angustifolius*, *L. albus*, and *L. luteus*. These will be key resources for building our knowledge of lupin genomes and genetic control of domestication and agronomic traits.

Many types of markers have been developed for lupin species, some of which (gene-based STS markers) are transferable between species. However, due to the large effort required to develop STS markers, there are still relatively few available. Strategies for generating many more markers must be developed to more adequately resource lupin genetic research. One strategy being employed in Poland and Australia is BAC-end sequencing of *L. angustifolius* BAC libraries (Foley

et al. 2008; Książkiewicz et al. 2008). STS markers can then be developed that exploit variability at microsatellite repeats found in some BAC-end sequences.

With the advent of next generation sequencing platforms (reviewed by Mardis 2008), other approaches for identifying large numbers of STS markers have now become feasible and affordable. While sequencing lupin genomes is still expensive and complex, large-scale sequencing of transcribed genes (also known as the transcriptome) in multiple genotypes is much cheaper and technically simpler. Barbazuk et al. (2007) were the first to describe this approach in a complex crop genome (maize). They used the Roche/454 genome sequencing technology to discover thousands of single nucleotide polymorphisms (SNPs) between two inbred parents of a maize mapping population. The recently reported method described by Maughan et al. (2009) provides a promising alternative whereby SNPs can be identified cheaply by sequencing multiple genotypes using reduced genomic complexity, barcoding and Roche/454 genome sequencing. These approaches could equally be applied to the parents of lupin RIL mapping populations and to the SNPs validated and mapped in the RIL populations. SNP array technology is also advancing with several platforms (such as the Illumina Golden Gate or Sequenom custom arrays) that could be used for high-throughput SNP genotyping. The new sequencing technologies could equally be used to develop other types of STS marker such as microsatellite markers; an approach of this type is currently being used to develop microsatellite markers for *L. luteus* (Maureira Butler and Salvo-Garrido personal communication). The availability of extensively expressed gene sequences would also expedite investigations of candidate genes implicated in traits or in biochemical processes of interest.

9.7.4 Association Mapping in Lupins

Association mapping (AM) is a powerful approach for identifying genes or genomic regions controlling complex traits. AM was pioneered in human and livestock genetics but is now becoming feasible in crop species as a consequence of recent developments in high-throughput SNP marker technology and in computational tools for exploiting population structure in crop species (Zhu et al. 2008). The AM approach is particularly attractive to animal and plant breeders because it increases potential genetic progress in existing breeding material and in germplasm collections without the need to custom-make populations as is the case in the conventional QTL approach. Crucially, AM encompasses more genetic diversity than conventional QTL mapping does and provides greater genetic resolution because it takes advantage of meiotic recombination in many lines over many generations.

Lupin breeding and genetics are well-placed to exploit the AM approach because fully-characterized germplasm collections and breeding populations already exist for the main agricultural lupin species. Good linkage mapping populations are also available, which are essential for providing map locations of markers used in AM and for the independent verification of significant associations identified by AM. The main limiting factor for implementing effective AM in lupin is the restricted availability of sufficiently large numbers of mapped STS markers required to fully exploit the association mapping approach. The SNP discovery and mapping approach described above may offer the best solution to overcome this limiting factor.

There are many benefits that AM could offer to lupin researchers and breeders. Basic research applications of AM include: identifying genes controlling traits of interest; determining the differences and commonalities of the domestication process in the different lupin species; and improving understanding of the genetic diversity in wild and domesticated populations. Breeders could benefit from newly identified marker-trait associations for MAS purposes, from a better understanding of the allelic diversity in their own breeding programs, and from enhanced genetic progress through incorporation of markers in the analysis of field trial data.

9.7.5 Integration of the Genetic and Physical Maps

Recently, cytogenetic mapping of lupins has been developed. It has been focused first on rDNA and some other repetitive sequence mapping. Currently, it concerns mainly the exploitation of a BAC library of the *L. angustifolius* nuclear genome (Kasprzak et al. 2006). BAC clones, including markers for some

valuable traits, have been selected from the library and used as molecular probes for BAC-FISH. Cytogenetic markers for specific chromosomes were developed from physically mapped single-locus BACs. Several markers, generated on the basis of BAC end sequences, were used for genetic mapping (Leśniewska and Naganowska 2009; Przysiecka et al. 2009). The work is aimed on the assigning of linkage groups to particular chromosomes and the complete integration of the physical and genetic maps of *L. angustifolius*.

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Chapter 10
Medicago

Iryna Sanders, Leonid Sukharnikov, Fares Z. Najar, and Bruce A. Roe

10.1 Introduction to the Legume Family

Legumes, second only to grasses as the most important agriculturally important family, include more than 650 genera and 20,000 species that grow on over 400 million acres, i.e., about 12% of the Earth's land, and contribute up to 27% of worldwide crop production and 33% of the human diet (Graham and Vance 2003). In addition to their economic importance, legumes play critical role in natural ecosystems since they have the ability to fix nitrogen via symbiosis with *Sinorhizobium* bacteria. This partnership allows the conversion of atmospheric inorganic N_2 gas into nitrogen compounds that are precursors to proteins and natural fertilizers. Since legumes produce three-times more protein than cereal grains and more ammonia fertilizer than the entire worldwide industrial production, they can occupy lands unsuitable for other plants (Graham and Vance 2003).

The importance of legumes is not limited to protein production and contribution to soil fertility as they also produce a broad range of natural products, secondary metabolites, which include flavonoids that are involved in symbiosis, disease/defense interactions, and plant developmental processes. This makes legumes an ideal model for both biochemical and genetic studies (Dangl 1998; McKhann et al. 1998; Constabel 1999). In addition, there is an expanding body of research regarding the healthful effects of flavonoids including their anti-cancer, anti-inflammatory, anti-aging, and cholesterol-reducing potentials (Woo et al. 2005).

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As the human population grows and urbanization increases, there is an increasing demand for higher levels of protein-rich foods. Since legume yields per acre lag behind those of cereals, new technologies are required to enhance legume protein levels. These include broader breeding programs, a better use of marker-assisted technologies, and an emphasis on disease resistance, enhanced nitrogen fixation, and tolerance to soil constraints to improve crop yield. The development of these new technologies will greatly benefit from the knowledge of a typical legume's genomic organization and its genomic features.

10.2 *Medicago* Genus as a Part of the Legume Family

The *Medicago* genus (medics) is an important part of the Mediterranean, southern European, Asian, and northern African flora. Plants of this genus occur in steppe meadows, along wood edges, and sometimes as a weed in fallow fields and gardens. Medics prefer reasonably fertile soils but not distinctly acid or alkaline. Excessive field moisture early in the season can significantly reduce medic stands (Clark 2007). This genus includes 83 species of flowering plants that belong to the legume family (Fabaceae), tribe Trifolieae. Most members of the family are low, creeping herbs. However, alfalfa (*M. sativa*) grows to a height of 1 m, and tree medick (*M. arborea*) is a shrub. This genus contains 20 perennial weedy species and 34 annual weedy species. Within the last group, some species present variability for their perenniality, for example, some populations of *Medicago lupulina* are pluriannual (Edwards et al. 2006). *Medicago* species are used as food plants by the larvae of some

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Lepidoptera species including Common Swift, Flame, Latticed Heath, Lime-speck Pug, Nutmeg, Setaceous Hebrew Character, Turnip Moth, and case-bearers of the genus *Coleophora* including *C. frischella* (recorded on *M. sativa*) and *C. fuscociliella* (feeds exclusively on *Medicago* spp). *Medicago truncatula* has natural genetic resistance against aphids and resistance against chewing insects due to jasmonic acid signaling and secondary metabolite production (Edwards et al. 2006).

Because of its extensive genetic resources, *M. truncatula* was selected as a model system that would serve as a reference for all members of this family (Bell et al. 2001). Since the genome organization of organisms from the same taxonomic group is fairly well-conserved, the information about the gene order and organization of one legume can be applied broadly to other legume species. *M. truncatula* has a small diploid genome (500 Mbp), can self-fertilize with a short seed-to-seed generation time, and is ideal for genetic studies because of its high transformation efficiency, the availability of the large collection of phenotypic mutants, and naturally occurring ecotypes (McKhann et al. 1998; Constabel 1999). In addition, the genus also offers a wide range of diversity. Some species are diploids, tetraploïds, or even hexaploïds (*M. cancellata*, *M. saxatilis*, and some populations of *M. arborea*). The chromosome number is often 8, but it can also be 7 (*M. constricta*, *M. polymorpha*, *M. praecox*, *M. rigidula*, or certain taxons of *M. murex*) (Prosperi et al. 1995).

10.3 Agricultural Usage

Medicago species (medics) are widely used in agriculture as they can produce almost as much biomass and N as clovers. Perennial medics are self-reseeding with hardy seeds that can take several years to germinate. This makes medics ideal for long rotations of forages and cash crops in the northern plains and in cover crop mixtures in the drier areas of California. Medics have high biomass productivity of 100 lb per acre in the Midwest under favorable conditions but have the potential for 200 lb per acre. Other agricultural benefits of *Medicago* spp. include conservation of soil moisture, protection from weeds due to quick spring regrowth, organic matter boost, erosion control due to hard-seeded tendency, drought tolerance and root penetration up to 5 feet deep, and tolerance to regular grazing and mowing. The annual medics are susceptible to diseases caused by *Rhizoctonia*, *Phytophthora*, and *Fusarium* (Clark 2007).

The best-known crop of the genus is alfalfa (*M. sativa*). Alfalfa is generally considered as one of the best forage crops for feeding ruminant animals because of its high nutritional value. Alfalfa leaves are protein- and vitamin-rich and low in cell wall concentration and, therefore, highly digestible. In contrast to leaves, stems exhibit low digestibility as a result of high concentrations of cell wall polysaccharides and lignin (Schnurra et al. 2007).

Several of the creeping members of the family (such as *M. lupulina* and *M. truncatula*) have also been used as forage crops. Alfalfa has higher stem crude protein concentrations than the *M. truncatula*, but both species have the same digestibility, providing support for the idea that alfalfa and *M. truncatula* stems are similar in cell wall structure (Schnurra et al. 2007). These characteristics make members of the *Medicago* genus very promising forage plant for improving pastures.

10.4 Karyotype and Cytogenetics of *Medicago* spp.

Most of the *Medicago* molecular cytogenetic studies have been done only on *M. sativa* and *M. truncatula*. A molecular cytogenetic map of *M. truncatula* was constructed on the basis of a pachytene DAPI karyogram in which all chromosomes were identified based on their chromosome length, centromere position, heterochromatin patterns, and the positions of three repetitive sequences (5S rDNA, 45S rDNA, and the MtR1 tandem repeat), visualized by fluorescence in situ hybridization (FISH) (Kulikova et al. 2004). The cytogenetic map was later integrated with molecular and genetic maps. The pachytene chromosomes allowed to distinguish large heterochromatic blocks with low gene density around the centromere from euchromatic arms rich in genes. It was determined that the total length of pachytene chromosomes is 406 μm, of which about 350 μm is euchromatic or estimated size of gene-rich region is 105 Mb of the 500 Mb *Medicago* genome.

M. truncatula chromosomes were divided into three groups. The longest chromosomes 1, 3, and 4

measure 60–68 µm and have centromere positions with centromere index (CI) values of 36%, 27%, and 30%, respectively. Chromosomes 3 and 4 are hard to distinguish because they have similar symmetrical heterochromatic regions, arm lengths, and centromere positions. MtR1 was used to distinguish chromosomes 3 and 4 (Kulikova et al. 2004). Three medium-sized chromosomes 2, 5, and 7 measure approximately 50 µm. Chromosomes 2 and 5 have median centromere positions (centromere indexes of 47 and 46%, respectively), whereas chromosome 7 has a submedian centromere (CI = 30%). In addition, chromosome 5 has a characteristic pattern of four pericentromeric heterochromatic knobs. In contrast, chromosomes 2 and 7 have only one knob on each arm. Furthermore, chromosome 5 contains the secondary constriction. Two smallest chromosomes 6 and 8 are 29 µm and 33 µm long, respectively, and vary in their chromatic patterns. Chromosome 6 has several heterochromatic chromomeres on both arms, whereas chromosome 8 has two larger heterochromatic blocks on either side of the centromere (Kulikova et al. 2004).

Based on FISH hybridization results, 5S rDNA loci are located on chromosomes 2, 5, and 6. A major 5S rDNA region occurs on the distal part of the pericentromeric heterochromatin of chromosome 5, on the arm containing a single heterochromatic knob. A second, smaller 5S rDNA region is located on the long arm of chromosome 2, close to the border of the pericentromeric heterochromatin. A third 5S rDNA site is present on chromosome 6. FISH with the 45S rDNA probe demonstrated a bright spot on the secondary constriction of chromosome 5, between two proximal heterochromatic knobs. The MtR1 tandem repeat was found in the pericentromeric regions of the chromosome arms 1-L, 2-L, 4-S, 7-S and 7-L, and 8-L (Kulikova et al. 2004).

The advanced molecular cytogenetics is also available for major subspecies of *M. sativa*. Based on the pachytene karyotype, the diploid ssp. *falcata* is similar to the diploid ssp. *coerulea* (Gillies 1968). The major differences of the pachytene karyotypes lays in the amount of heterochromatic DNA, and it was observed that diploid ssp. *falcata* contains a lower amount than does ssp. *coerulea*, results also supported by mitotic chromosome C- and N-banding studies (Bauchan and Hossain 1999).

A majority of the pachytene chromomeres in *M. sativa* ssp. *falcata* are located on either side of the centromeres, and telomeric knobs are not observed except when an entire arm is chromatic. The somatic chromosome karyotype of *M. sativa* ssp. *coerulea* is represented by one pair of satellite chromosomes (chromosome 8), four pairs of submetacentric chromosomes (chromosomes 1–4), and three pairs of short metacentric chromosomes (chromosomes 5–7). The nucleolar organizer regions (NOR) of the alfalfa genome is located on chromosome 8; the hallmarks of this karyotype are the SAT chromosomes.

FISH data with the number of nuclear ribosomal loci present in *Medicago* are only available for eight species including three out of 12 sections of the genus (Table 10.1; Rosato et al. 2008), making it difficult to understand the evolutionary dynamics within

Table 10.1 Cytogenetic features (chromosome number and ploidy level) and the number of reported 45S and 5S rDNA loci in *Medicago truncatula*

	Medicago spp.	Chromosome number	45S rDNA	5S rDNA
Section *Dendrotelis*	*M. arborea*	$2n = 32$ ($4x$)	1	2
	M. strasseri	$2n = 32$ ($4x$)	1	2
	M. citrina	$2n = 48$ ($6x$)	4	5
Section *Medicago*	*M. sativa* subsp. *coerulea*	$2n = 16$ ($2x$)	1	2
	M. sativa subsp. *glomerata*	$2n = 16$ ($2x$)	1	2
	M. sativa subsp. *falcata*	$2n = 16$ ($2x$)	2	2
		$2n = 32$ ($4x$)	2	4
	M. sativa subsp. *sativa*	$2n = 32$ ($4x$)	2	4
	M. marina	$2n = 16$ ($2x$)	1	2
Section *Spirocarpos*	*M. truncatula*	$2n = 16$ ($2x$)	1–2	1–3[a]
	M. lesinsii	$2n = 16$ ($2x$)	1	1
	M. murex	$2n = 14$ ($2x$)	1	1

[a]One of the 5S rDNA loci is collinear with a 45S rDNA locus (after Rosato et al. 2008)

Table 10.2 Summary of *Medicago truncatula* genes and other genome elements

Chromosome number	No. of predicted genes	No. of single exon genes	No. of predicted exons	Gene density (%)	Repeat sequences (%)	Structural RNAs bp	No. of tRNAs	No. of transposons
0	1,995	560	6,051	10.85	7.91	6,286	31	130
1	5,719	1,387	20,288	16.45	9.74	8,118	84	227
2	5,402	1,427	18,625	17.68	10.93	5,013	60	220
3	7,083	1,801	23,999	17.21	10.89	5,355	98	290
4	7,674	1,984	26,467	17.71	11.21	7,972	85	338
5	8,074	2,053	27,258	19.19	9.23	96,980	101	233
6	3,672	964	10,892	17.41	16.38	1,812	28	264
7	6,097	1,560	20,840	16.77	11.45	4,880	65	239
8	6,278	1,603	22,260	16.25	10.68	4,548	64	284

Chromosome 0 includes all genes that could not be mapped to any other chromosome

Medicago, as has been done in other genera (Mishima et al. 2002).

The diploid species of *Medicago* show a single 45S locus and two 5S loci with an exception of *M. truncatula* (Table 10.2; Rosato et al. 2008). Tetraploid species of *Medicago* showed the number of rDNA signals that are consistent with a diploid-doubled to tetraploid model of polyploidization (two of 45S rDNA and four of 5S rDNA). However, species from section *Dendrotelis* do not fall in the same pattern. It looks like the tetraploid taxa of section *Dendrotelis* (*M. arborea* and *M. strasseri*) have experienced a diploidization event through physical loss of the sequences and not just loss of function. However, a simple chromosomal diploidization of rDNA loci cannot explain why the hexaploid *M. citrina* showed the highest number of rDNA loci reported in the genus so far. The possible explanation can be elimination, silencing, or rearrangement of rDNA genes that occurred early after polyploidization events (Skalicka et al. 2005).

10.5 Phylogenetic Relationship and Evolution of *Medicago* Species

The first taxonomic analysis of the *Medicago* genus was performed more than 30 years ago (Lesinš and Lesinš 1979). Later, the comparative analysis on species has been done using multiple methods such as cytology, isozymes, and DNA polymorphism, in addition to morphological and geographical data. We have discussed the most recent studies concerning the relationship within the genus (Table 10.3).

Using restriction fragment length polymorphism (RFLP) band patterns detected by chloroplast DNA variation, Valizadeh et al. (1996) estimated genetic distances among nine species of *Medicago* and clustered them according to the level of distances between markers. They showed a significant genetic similarity between *Medicago* species. The most similar were *M. truncatula* and *M. littoralis*, and the most distantly related was *M. lupulina*. Based on RFLP, all the studied species were divided into four groups (1) *M. truncatula*, *M. littoralis*, *M. sativa*, *M. scutellata*; (2) *M. orbicularis*, *M. radiata*; (3) *M. rigidula*, *M. minima*; and (4) *M. lupulina*.

As it would be expected, genetically distant *Medicago* species can have different life form, mating system, and seed dispersal. For example, comparing *M. lupulina* and *M. ruthenica*, the former is an annual, biennial, or occasionally short-lived perennial, predominantly self-fertilizing, and widely distributed plant, whereas the latter is long-lived perennial, outcrossing, and much more narrowly distributed plant (Yan et al. 2009). They also differ in their genetic variation and level of heterozygosity. *M. ruthenica* populations possessed a larger average number of private alleles and a higher allelic diversity than *M. lupulina* populations (Yan et al. 2009).

Based on molecular variance analysis, the majority of genetic variation in *M. lupulina* occurred among populations and in *M. ruthenica* within individuals. However, the minimum partitions of genetic variation in *M. lupulina* were observed within individuals while they varied among populations in *M. ruthenica* (Yan et al. 2009).

The estimate of population differentiation showed a much higher population genetic differentiation in

Table 10.3 Comparative analysis of the sequenced genomes

Organism	Genome size (Mbp)	No. of genes	Gene size (kbp)	Gene density (1 gene/kbp)	Av. exon length (kbp)	Av. intron length (kbp)	Reference
Arabidopsis	125	26,500	2.0	1/4.5	217	157	Sclep et al. (2007)
Rice	400	45,000	2.6	1/9.9	254	413	Li et al. (2006)
Maize	2,400	~50,000	4.0	1/43.5	259	607	Haberer et al. (2005)
Lotus	470	40,000	2.5	1/9.1	297	377	Young et al. (2005)
Medicago	500	50,000	2.7	1/10.0	266	375	Young et al. (2005)

M. lupulina than in *M. ruthenica*. Genetic and geographic distances for both species have a strong positive correlation, suggesting that territorial separation played an important part in defining their genetic differences. Although these species are geographically and, therefore, genetically separated, there are some evidences of positive gene flow between populations of both species as around 15% of individuals differ from their parental species (Yan et al. 2009).

Within largely herbal *Medicago* genus, there is a section *Dendrotelis*, woody medics including *M. arborea*, *M. citrina*, and *M. strasseri*. Although the woody species are morphologically different from other medics, phylogenetically they are not isolated from the *Medicago* genus as a whole (Rosato 2008). Although all woody species are polyploids (Table 10.2), the tetraploid *M. arborea* and *M. strasseri* have only single 45S rDNA locus and two 5S loci as diploid members of the genus, suggesting that a diploidization of ribosomal loci happened through physical loss of the sequences but not of function. Among woody taxa, the pair *M. arborea–M. strasseri* shows strong morphological and genetic similarities, *M. citrina* is genetically divergent from them having a high number of 45S rDNA loci and specific organization of NORs. The origin of *M. citrina* is unclear; it is believed that this species has originated through autopolyploidy from other woody species of section *Dendrotelis*, hybridization between *M. arborea* and/or *M. strasseri*, and an unrelated diploid species from section *Medicago* (Rosato 2008).

The phylogenetic relationship between *Medicago* species was analyzed by Bena (2001) on the basis of a molecular phylogenetic approach using the internal and external transcribed spacers of 53 sequenced *Medicago* species and 10 medicagoid species. His version of *Medicago* species relationship is represented on Fig. 10.1. He also proposed to place morphologically atypical *Medicago* species (such as *M. radiata* and *M. platycarpa*) in *Medicago* rather than in a new genus.

The same research group (Bena et al. 2005) made an attempt to compare the phylogenetic trees of *Medicago* and *Rhizobia* species to understand if the symbiotic association influences the diversification of the *Medicago* genus. Several different symbiotic groups were specified in this genus. *M. radiata*, *M. brachycarpa*, *M. monantha*, *M. orthoceras*, *M. noeana*, and *M. rigiduloides* are able to fix nitrogen only when in association with the Rhizobia strain USDA 1623 (SG1). However, using molecular phylogenetic method, these species belong to two different branches (see Fig. 10.1).

The two closely related species *M. laciniata* and *M. sauvagei* (SG2) fixed nitrogen only when associated with the strain USDA1635. Eight species (*M. arabica*, *M. granadensis*, *M. intertexta*, *M. ciliaris*, *M. muricoleptis*, *M. tenoreana*, *M. shepardii*, and *M. polymorpha*) all fixed nitrogen in association with *Sinorhizobium medicae* strains but phylogenetically belong to four separate branches. No strict specificity between *Medicago* and the bacteria species was found. Based on a visual inspection of the *Medicago* and Rhizobia phylogenies, a possible punctual reciprocal event of codivergence was suggested (Bena et al. 2005). Although the *Medicago* genus diversification supposedly occurred from 65 to 2 million years ago (Mya) (Lesinš and Lesinš 1979) and Rhizobia diversification started 10 Mya, there is a possibility that both organisms have undergone chromosomal co-speciation (Bena et al. 2005).

10.6 Comparison of *Medicago* Genome Features with Other Legume Genomes

Although all legumes share many common genomic features, there are unique differences. For example, *M. truncatula* and *Lotus japonicus*, the two model plants that represent cold climate and tropical

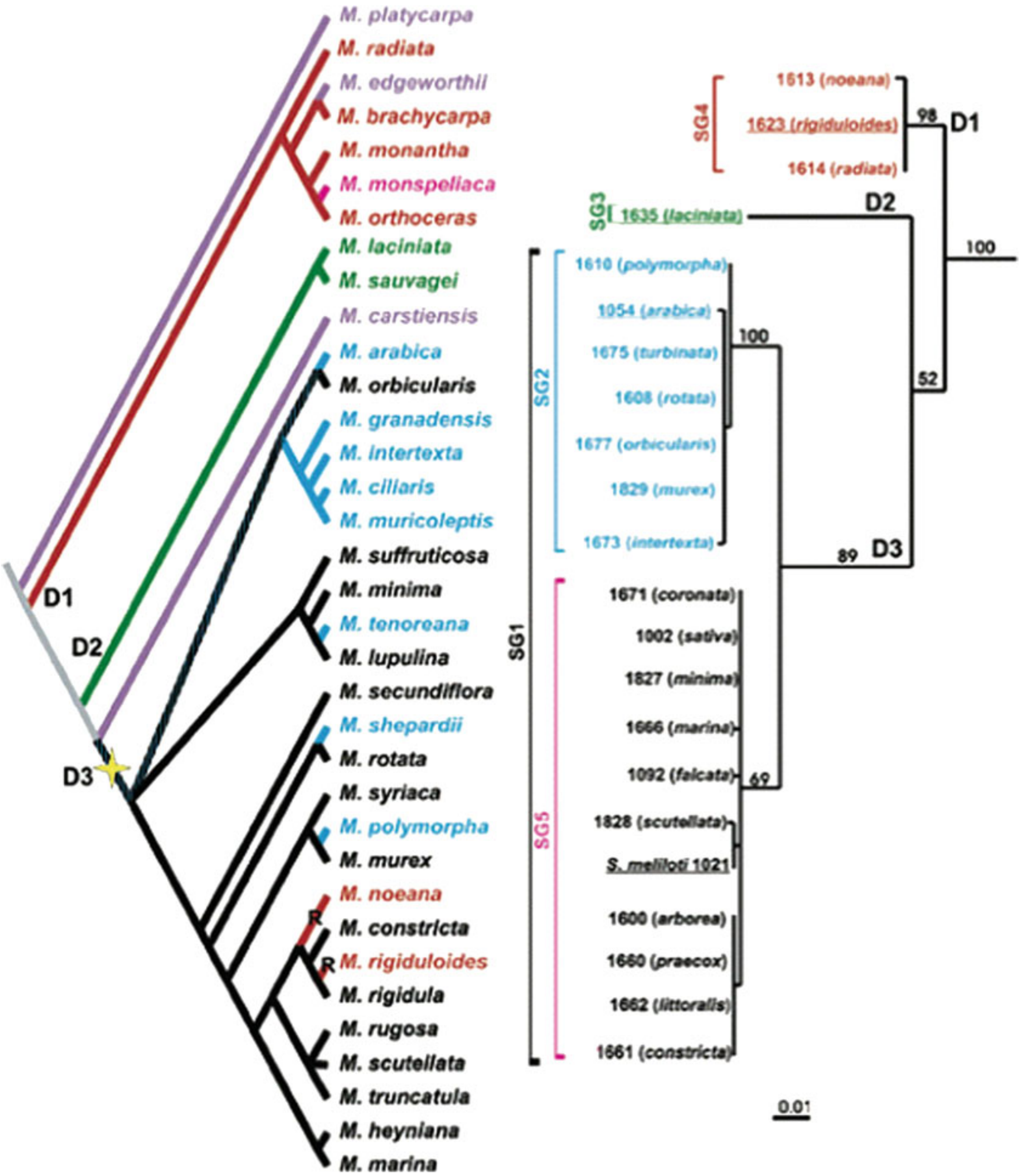


Fig. 10.1 Symbiotic association between *Medicago* species (*left*) and *Sinorhizobium* strains (*right*) (Bena 2001). The *same colors* between *Medicago* species and bacterial strains indicate an efficient nitrogen fixation between these species. The five specificity groups (SG) were defined based on the fixation test's results, with the corresponding colors. *Medicago* species in *black* performed an efficient symbiosis with strains from *S. meliloti* type 2, *S. meliloti* type 3, and *S. medicae*. *Medicago* species in purple did not fix nitrogen with any of the strains tested. The colors of the branches in the plant phylogeny indicate the putative symbiotic state along this branch during the evolution of the genus. *Dashed branches* reflect uncertainty in the determination. Strains *underlined* are the one that have been used in the fixation tests. Plant phylogeny branch lengths are arbitrary. A *yellow star* on the plant phylogeny indicates a main modification of symbiotic association, whereas the "R" indicates putative reversion of specificity. The similar "D1, D2 and D3" in the two phylogenies indicate corresponding divergence events between the two trees

legumes, respectively, have substantial macrosynteny and microsynteny as determined by marker-based comparisons (Young et al. 2005). Both legumes also have a similar genome size around 450–470 Mbp, similar number of genes 35,000–40,000, a similar gene density of one gene per 6.3 kb for *L. japonicus* and one gene per 6.7 kb for *M. truncatula*, and have 82% conserved genes. Their genes mainly are located in euchromatic regions that comprise the chromosome arms and are not found in heterochromatin of centromeres and pericentromeres (Young et al. 2005). *Medicago* and *Lotus* have significant synteny despite that they are relatively distant relatives and the synteny is interrupted by chromosomal rearrangements that is reflected in the differences in their chromosome number. The observed conserved microsynteny is characterized by 80% of the close homologs that have had the same gene order and transcriptional orientation, similar to that observed in humans and mice. Species-specific tandem duplication of genes accounts for an additional 12–17% divergence of gene content. Considering their similarity in gene size, order, and orientation, they probably have undergone similar genome development. However, these two legumes differ in both the diversity and organization of their repeat sequences. Both genomes encode specific novel repeat families that are clustered into large groups (Young et al. 2005). In *M. truncatula*, MtR3 repeats of up to 1 Mbp are found within centromeres, whereas MtR1 and MtR2 repeats are present mainly in pericentromeric regions. However, *L. japonicus* has its own repeats, LjRE1, LjRE2, and LjTR1 that are shorter and located in chromosome arms, pericentromeric regions, and chromosome knobs, respectively (Young et al. 2005). Interestingly, both legumes have similar retrotransposon and transposon compositions.

Comparison of *Medicago* genome features with those of other legume relatives of larger genome size also reveals a high degree of synteny. Diploid pea (*Pisum sativum*) has 5–10 times larger genome than *Medicago*, but they show a strong conservation of gene order (co-linearity). Five *Medicago* linkage groups (1, 4, 5, 7) are completely colinear with pea linkage groups 2, 7, 1, 5, and 4, respectively. The other *Medicago* and pea linkage groups have more chromosomal rearrangements, but co-linearity is maintained. Both plants also display more than 85% of gene homology. These two legume species represent closely related tribes with differences in genome sizes due to multiple transposition events and accumulation of polymorphic transposon insertions in pea that occurred after pea and *Medicago* diverged (Kalo et al. 2004).

In contrast, the relationship between *Medicago* and soybean is more complex because soybean underwent polyploidization followed by gene loss and segmental reshuffling that makes it difficult to identify syntenic segments in these two plants. Some observations show that between 30 and 54% of soybean contig groups combined in 11 blocks have microsynteny to *M. truncatula*. Around 81% of soybean genes have their homologs in *Medicago* genome, and 70% of their genes are conserved and colinear. Although all legume genomes underwent varying degrees of duplications as they evolved, at least two duplications observed in soybean that dramatically changed its genome organization because approximately 25% of the genes are physically clustered in soybean, paralogs recovered from duplicated genes share 86–100% sequence identity, and more than 90% of the non-repetitive sequences are present in more than two copies, with the average chromosomal segment being duplicated approximately 2.55 times. *Medicago* also underwent a number of duplications because the *M. truncatula* genes in orthologous regions are closely related to soybean based on synonymous substitution levels, percent identity, and extent of synteny between *Medicago* and soybean lineages. Although soybean genome is more than twice larger than *Medicago* genome, both have very compatible gene densities in syntenic blocks that can be considered as an additional evidence for possible gene clustering in *Medicago* similarly to soybean (Mudge et al. 2005).

10.7 *M. truncatula* Sequencing Initiative

M. truncatula genome sequencing was done in four sequencing centers: Bruce Roe et al., The University of Oklahoma; Chris Town et al., The Institute for Genomic Research (TIGR); Jane Rogers et al., Sanger Centre; Francis Quétier et al., Genoscope (Young et al. 2003). Originally, the *M. truncatula* sequencing project began as a pilot project with a whole-genome shotgun study that generated 25,000 sequence reads (Roe and Kupfer 2004). As the cytogenetic mapping improved, several BACs also were chosen for

sequencing as they contained legume resistance genes with high biological interest (Kulikova et al. 2001). Because of the high level of repeat sequences in the whole-genome shotgun, a fingerprint-based physical map and the availability of approximately 177,000 BAC-end sequences, a BAC-by-BAC approach was proposed to sequence the *M. truncatula* gene space (Nam et al. 1999). To create a genome physical map, BAC DNA was isolated, digested with *Hind*III, and end-labeled at *Hind*III sites with radioactive dNTPs. The DNA fragments were subjected to polyacryamide gel electrophoresis and exposed to X-ray films. The fingerprints were scanned into image files and edited to exclude clones, which could not provide sufficient information for contig assembly. The physical map has been assembled using the fingerprint contig (FPC) software and anchored to the genetic map by means of BAC-based genetic markers (Mun et al. 2006). For sequencing, clones were selected based on a minimum overlapping minimal tiling path. Here, initial "seed" BACs were confirmed by physical mapping and sequenced (Thoquet et al. 2002), and then new BAC clones were chosen by extending the finished BACs using the minimal tiling path. Because of the large number of repeats in plants, 10–20% of FPC map was incorrect. Therefore, at later stages of the project, the sequencing groups switched from FPC maps to more extensive use of BAC end sequences to extend and eventually join contigs to form pseudo-chromosomes (Choi et al. 2004). After screening out repeats, the sequence of more than 95% of the euchromatic regions of the *M. truncatula* genome is now rapidly being reached. Approximately, 75% of the *M. truncatula* genome sequencing was done using the Sanger dideoxynucleotide method with fluorescent terminators, while the remainder was finished using the combination of this method and the newer 454 based pyrosequencing approach.

10.7.1 M. truncatula *Sequencing Using the Fluorescence Terminating Method*

The *M. truncatula* large-scale BAC DNA isolation and BAC library construction is outlined in Fig. 10.2.

Sequencing was performed using either the BigDye or ET terminator kits, which contains Taq polymerase buffer, AmpliTaq Fs DNA polymerase, the four dNTPs, and the four dye-labeled dideoxynucleotide terminators. During incubation, the dideoxynucleotide terminator containing a fluorescent dye, a fluorescine-derivative (donor dye) connected to a dRhodamine-derivative (acceptor dye), resulted in a nested fragment set that then was electrophoretically resolved on either an ABI 3700 or ABI 3730 capillary sequencers. Here, the capillaries were automatically filled with flowable gel mix POP-5 prior to electrokinetically injecting the sequencing reaction products that were dissolved in 0.1 mM EDTA, pH 7.6. Electrophoresis was performed at 6.5 kV for 2.5 h and the dye-labeled terminators were excited by an argon ion laser source of PE Applied Biosystems DNA sequencer. Energy emitted by the donor dye excited the electrons in the dRhodamine acceptor, and emitted light was detected by a CCD camera in the ABI-3700 or ABI-3730, when they return to their ground state (Rosenblum et al. 1997). Because of the small capillary inner diameter (50 μm), the temperature gradient within the tube was minimized and the heat was dissipated rapidly in the applied high electric field. The fluorescence data was collected automatically and analyzed using ABI base caller on Dell-PC computer and then transferred to a Unix-based SUN workstation.

10.7.2 454 Pyrosequencing Technology

454 pyrosequencing technology emerged in early 2005 and was successfully adapted by *M. truncatula* genome sequencing project (Margulies et al. 2005). Pyrosequencing is a sequencing method based on synthesis (Lindström et al. 2004). On a first step, a sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template and incubated with the enzymes: DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates: adenosine 5′ phosphosulfate (APS) and luciferin. Next, the first of four deoxynucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxynucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. On the third step, ATP sulfurylase quantitatively

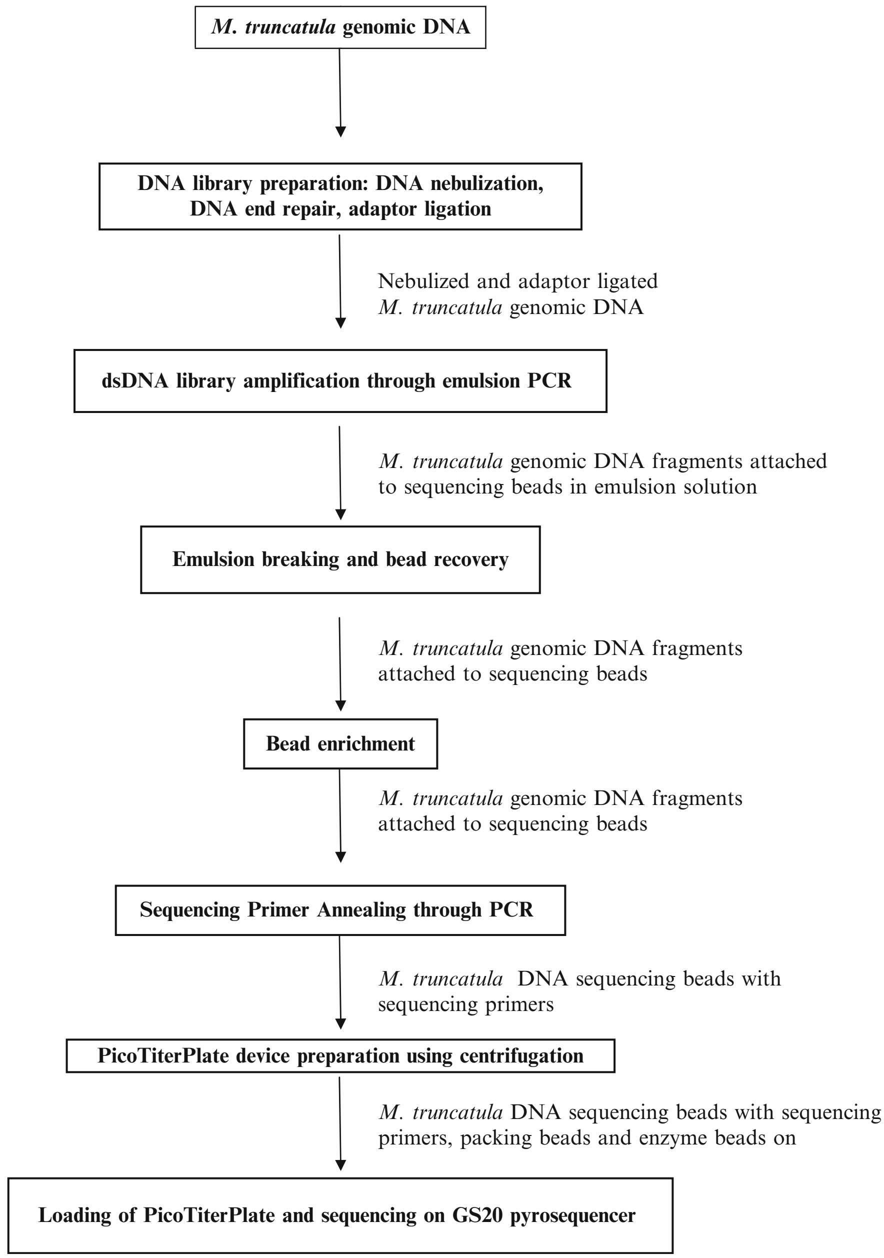


Fig. 10.2 Detailed scheme of *M. truncatula* sequencing

converts PPi to ATP in the presence of adenosine 5′ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram™. Each light signal is proportional to the number of nucleotides incorporated. On the fourth step, apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added. Addition of dNTPs is performed one at a time. Deoxyadenosine alfa-thio triphosphate (dATP S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase but not recognized by the luciferase. As the process continues, the

complementary DNA strand is synthesized and the nucleotide sequence is determined from the signal peak at each base addition.

When it became clear that the total gene space of the *M. truncatula* genome was significantly larger than initially predicted, we began to implement a BAC pooling approach that reduced the overall sequencing costs and improve sequencing efficiency. Individual BAC clones were grown to stationary phase in 100 ml cultures. Then 20 ml of each BACs were pooled such as each pool contained Z number (were 9, 10, 11, 24) of BACs representing Z × Z horizontal array. BACs were also pooled vertically where the first column of Z × Z horizontal array was a first vertical pool, etc. The BAC DNA was then isolated from eachpool using the standard method described above, and shotgun libraries are generated using protocols for the dye-terminating and pyroseqencing approaches. The resulting data, therefore, contains the shotgun sequence from each of the Z BAC-containing pools. For the assembly phase, the overlapping adjacent contig sequence for each extending BAC was added to each pool along with the individual BAC-end sequences for each pool member, low coverage pooled BAC shotgun ABI 3730 data, and 454-generated pooled paired-end reads after assembly using the 454 Newbler assembler. This combined 454 (shotgun and paired end-data) and ABI 3730 (shotgun and BAC endread) and then was assembled using Phred/Phrap. Data obtained from sequencing of horizontal pools was compared with the vertical pool sequences, and the intersection of both pools consisted of data from the individual BAC in that intersecting well of the Z × Z array (Csuros et al. 2003).

10.7.3 Sequence Assembly

The data from the sequencers was transferred to a project directory on the SUN station using the file transfer protocol (ftp). The chromatograms produced by the sequencers for each reaction, called the trace files, were analyzed by Phred software (Ewing et al. 1998) that determines the base sequence and assigns quality values to each base. Here, Phred first determines where the peaks would be centered if there were no factors shifting the peaks from their calculated locations. Then, the center of actual peaks is located for each trace (Ewing et al. 1998), and the traces surrounding each called base are evaluated using quality value parameters to assess the trace quality and uses trace parameters to produce error probabilities associated with each called base. Phrap, the actual sequence assembly software, then uses the information provided by Phrep together with the read alignments to assign an error probability to each base of the inferred underlying sequence (consensus sequence) of the clone and construct the contigs (Gordon et al. 1998).

Contigs were viewed using the Consed contig viewer program (Gordon et al. 1998) that uses the quality files produced by Phred and the output assembly files generated by Phrap to create an accurate and interactive view of the data. Consed also shows the individual reads of the consensus sequence for each contig that are associated with the Phred quality files with white background and capital letters representing the high quality reads and a dark shade and low case letters representing the poor quality of data. Consed also can display the repeats in the sequence data and represent them by green shades as well as chimerical clones that are shown in red (Gordon et al. 1998).

10.7.4 Data Analysis and Annotation

Analysis and annotation of *M. truncatula* genome began once sixfold coverage was obtained. Initially, BAC sequencing data is run through RepeatMasker, a program developed by A. Smit and P.Green (*http://www.repeatmasker.org*) that screens DNA sequences for interspersed and low complexity DNA sequences. RepeatMasker by masking intersperced repeats in genomic sequences avoids spurious matches during databases searches. After repeat masking, genomic sequences were screened by tRNA – ScanSE to predict all tRNAa and BLASTN to predict all rRNAs. tRNAscan-SE scans against transfer RNA (tRNA) genes, which are also called non-coding RNA (Lowe and Eddy 1997) based on known conserved tRNA secondary structural features, such as common sized loops and stems and conserved nucleotides, such as the TTC in loop VI. The tRNAscan-SE program can detect 99–100% of tRNA genes as well as tRNA-derived repetitive elements and tRNA pseudogenes.

For gene prediction, Genscan and Fgenesh were used. These programs predict the transcriptional

units based on different algorithms/models. Genscan incorporates a description of the basic transcriptional, translational, splicing signals, length distribution, and compositional features of exons and introns, and creates the most likely gene structure and composition model of the genomic DNA for a given organism (Burge and Karlin 1997). Under this probabilistic model, each possible gene structure is assigned as a probability where exons are described as "optimal exons" and the translation products of the corresponding "optimal genes" are displayed as predicted peptides. Since Genscan was designed mainly for humans and vertebrates and usually under predicts genes for plants, Fgenesh also was used to identify genes in *M. truncatula* since it was a hidden Markov model and is considered to be more accurate and faster than Genscan (Solovyev et al. 1995). The combination of both programs allowed predicting genes on chromosomes with the highest accuracy. Also, because FgeneSH is trained on full-length cDNA, it has higher specificity and sensitivity, misses fewer exons, and predicts fewer wrong exons than Genscan (Fig. 10.3).

Then, the *M. truncatula* genome was searched for open reading frames (ORFs) that were identified after a BLASTN (Altschul et al. 1997) homology search against the GenBank NR database after gene prediction using FgeneSH (Burge and Karlin 1997) trained on *M. truncatula* full-length cDNAs and Genscan (Solovyev et al. 1995). The BLAST algorithm determines a statistically significant alignment based on high-scoring pair aligned words. Here, BLAST scans the database for these words (typically of length three for proteins) that score at least the default T value when aligned with words within the query sequence. Any aligned word pair satisfying this condition is called a hit, and each hit with the E value below the threshold is recorded. These "hits" are then grouped based on K-tuple and displayed (Altschul et al. 1997).

As this project progressed on, International Medicago Genome Annotation Group (IMGAG) was formed. The schems for the IMGAG is: BAC sequences are deposited by sequencing centers in EMBL or Genbank. The Institute for Genome Research (TIGR) retrieved the Munich Information Center for Protein Sequences (MIPS) phase 3 BACs that were analyzed by PASA (Haas et al. 2003) and Fgenesh/Mt, (PASA including MENS peptides and Mt ESTs); then this annotation was posted on the TIGR FTP site. The Toulouse sequencing center captured this information and analyzed the data using the gene-finding program, Eugene (Foissac et al. 2003). Since TIGR assigned preliminary protein descriptions using Interpro, and Tigrblast, if no significant similarity was found, the tag "no description assigned" was added. The BAC sequences and TIGR annotation then were combined, and this annotation was made publicly available (Cannon et al. 2005).

Proteins with no homology or homology below the E-value threshold were analyzed further for common patterns in the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways database, and the eukaryotic orthologous groups (KOG) and Pfam databases. If a protein sequence was distantly related to a known protein, a motif-based search using the Motif program at the Bioinformatics Center Institute for Chemical Research at Kyoto University, and protein pattern libraries such as Pfam (Bateman et al. 2002) and Prosite (Hofmann et al. 1999) were employed. Pfam allows searching for many common protein domains and families and link protein structure and functions, while the Prosite database consisting of biologically significant patterns and profiles can determine homology to known protein families (if any) or to known protein domain(s).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2000) contains the GENES database with gene catalogs for all completely and partially sequenced genomes, the PATHWAY database containing graphical representations for metabolism, membrane transport, and other cellular processes supplemented by a set of ortholog group tables for the information about conserved subpathways (pathway motifs), and the LIGAND database that provides the information about chemical compounds, enzyme molecules, and enzymatic reactions. KEGG also provides Java graphics tools for browsing genome maps, comparing two genome maps and manipulating expression maps, as well as computational tools for sequence comparison, graph comparison, and path computation, features that were useful to predict and complete metabolic pathways when BLAST homologs were absent.

As mentioned above, the euKaryotic Orthologous Groups (KOG) database (Tatusov et al. 2003), is a useful classification system for eukaryotic genomes based on relationships of genes with similar functional domains. The underlying premise of this database is that orthologs have sequence features that are more similar to each other than they are to any other protein in its genomes and thus can be joined to form clusters.

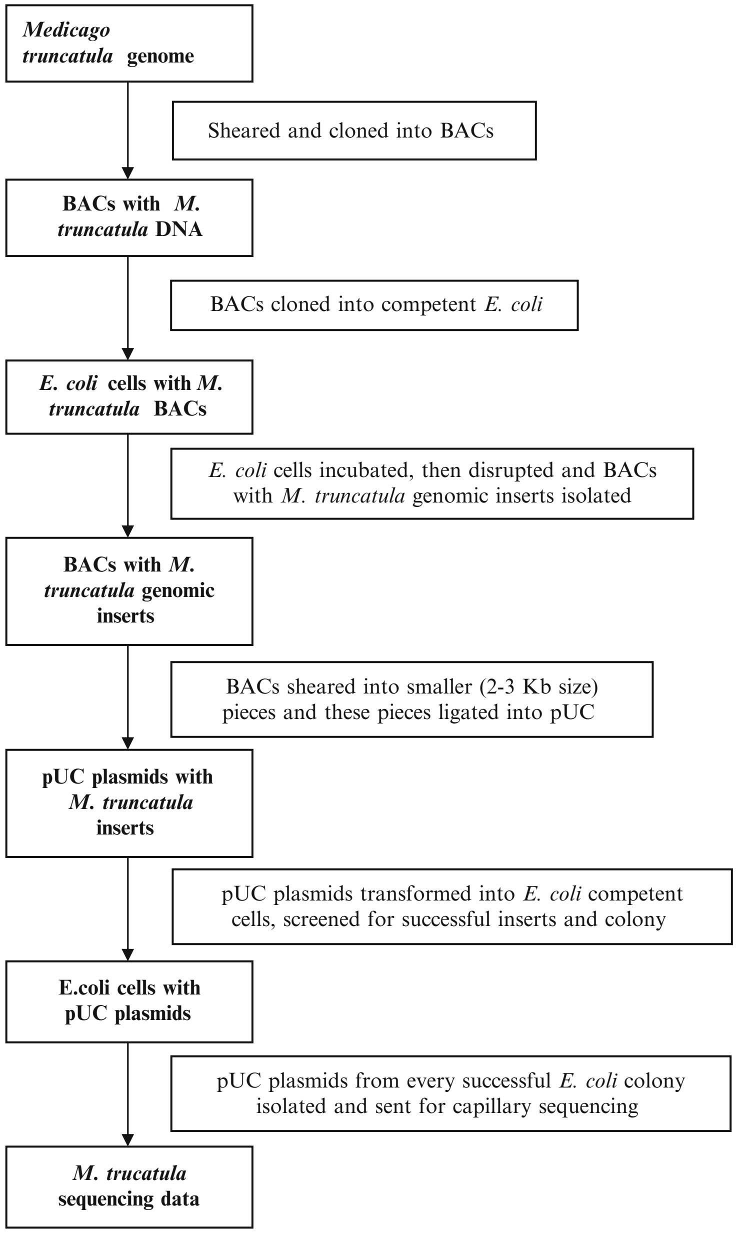


Fig. 10.3 BAC pooling strategy combined with 454 data collection based approach for genomic DNA sequencing

10.8 Sequencing Statistics for the *M. truncatula* Chromosomes

M. truncatula encodes approximately 52,000 genes including more than 13,000 single exon genes, 2,225 genes encoding transposable elements (Table 10.2), spanning approximately 176,680 exons. The average gene density in the euchromatic region is 1 gene per 4.4 kbp. *Medicago* gene has an average size of 2.7 kbp with a predicted peptide of 239 amino acids. Genes comprise approximately 16.6% of euchromatic region of the *Medicago* genome with the highest percentage on chromosome 5, which has 19.19% of genes.

The euchromatic region of the *Medicago* chromosomes contains on average of 10.9% repeated sequences, chromosome 5 has the lowest number of repeats and chromosome 6 has the highest (Table 10.2). Chromosome 5 also has the largest number of small RNAs, more than ten times of the amount of small

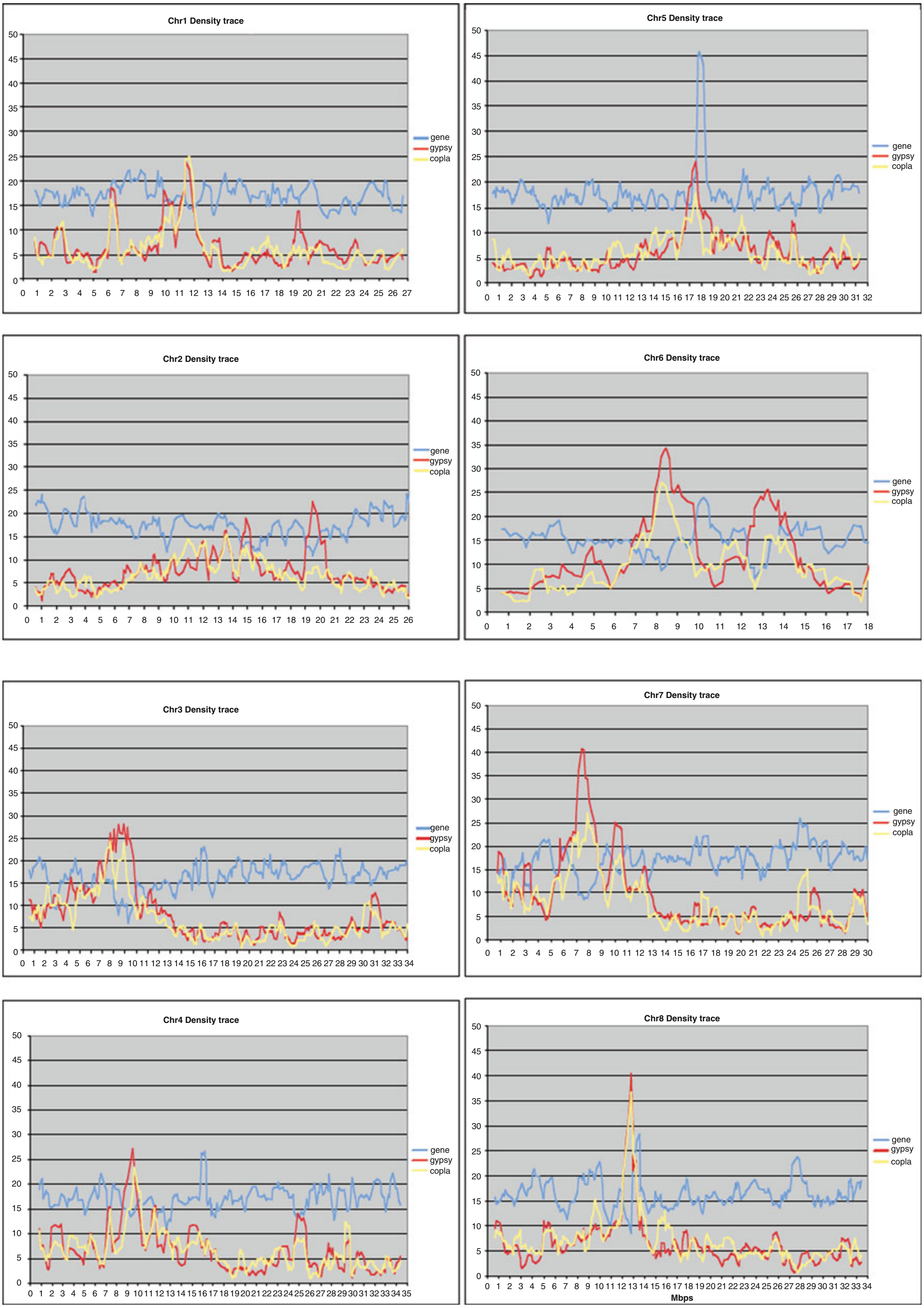


Fig. 10.4 Transposon and gene density on each of the eight *Medicago truncatula* chromosomes

RNAs on other chromosomes. The majority of these RNAs are rRNAs. Also, chromosome 5 is the only chromosome that contains rRNAs. Chromosome 5 has the largest amount of tRNA. *Medicago* chromosomes 5 and 6 have unusual gene densities; the former has the elevated gene density while the latter displays the reduced gene density. The rest of the chromosomes have relatively high and homogenous gene density. Also, chromosome 6 has an elevated level of transposons (Fig. 10.4).

10.9 *M. truncatula*: Importance for Gene Analysis

The legume family is extremely diverse, including tropical and temperate species from grain and forage crops to trees and herbs. Most legumes have large and complex genomes and therefore are unsuitable for detailed genomic research. *Arabidopsis*, another dicot, cannot serve as a model for legumes because of its lack of symbiosis, it cannot form endomycorrhizae, and it has very low levels of nitrogen-rich compounds and secondary metabolites. Fortunately, since the genome organization of organisms from the same taxonomic group is fairly well conserved, the information about the gene order and organization of one legume can be applied broadly to other legume species. This knowledge of synteny among legume genomes resulted in choosing *M. truncatula* as a model system that would serve as a reference for all members of legume family (Bell et al. 2001). The knowledge on *M. truncatula* genome organization would greatly facilitate gene discovery among related species.

The combined genetic, phylogenetic, and genomic information demonstrate extensive conservation of gene order and orthology between crop and model legumes and also reveal how these genomes vary structurally (Choi et al. 2004). For example, genomes of *M. truncatula* and *P. sativum* indicate a high level of conservation, although the latter has ten times larger genome and one less chromosome. That one less chromosome in *M. truncatula* corresponds to the highly heterochromatic chromosome 6, which has a low frequency of non-RGA EST markers. However, parallel analyses conducted by Kalo et al. (2004) suggest that *M. sativa* chromosome 6 has synteny with PsLGVI and PsLGVII regions in the pea genome. This observation helps us to detect the chromosomal fission/fusion events potentially responsible for the reduction of chromosome number in *P. sativum* (Choi et al. 2004).

L. japonicus and *M. truncatula* genomes represent the two best-characterized legumes and are always compared to each other due to recent divergence and sister-clade relationship. Although they share large fragments of genetic macrosynteny and sequence microsynteny, there is still a significant divergence between these two legume genomes due to the insertion/deletion of genes, tandem duplication of genes, and a unique distribution of mobile DNAs (Choi et al. 2004). Chromosomal rearrangements in legumes increase with phylogenetic distance effecting macrosynteny between species. Small regions of colinearity observed between *M. truncatula* and *P. sativum* or between *M. truncatula* and *L. japonicus* helps us to understand why it is difficult to identify macrosynteny between *M. truncatula* and soybean despite the relatively large number of genetic markers used for comparison. It is possible that recent duplication and low rates of polymorphism in the soybean genome contributed to that. Nevertheless, there is a significant conservation of microsynteny between *M. truncatula* and soybean (Cannon et al. 2003).

It was also observed that not only functionally conserved genes but also large and rapidly evolving gene families occupy syntenic positions across the diversity of legume species (Choi et al. 2004). Therefore, despite current challenges, the comparative genomics can be practically applicable in crop improvement.

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Chapter 11
Phaseolus

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11.1 Introduction

Common bean (*P. vulgaris* L.) has been grown on more than 12 million ha and constitutes the most important food legume for direct consumption by more than a billion people worldwide. Beans have been a very important source of protein and calories for Latin America, Africa, and Asia. The world annual production is about 9 million metric tons. Production occurs in a wide range of cropping systems and diverse environments in the Latin America, Africa, Middle East, China, Europe, United States, and Canada. In Latin America and Africa, beans have been primarily cultivated on small farms with few purchased inputs, in association with other crops. The species is native of the Americas, and after domestication, it has spread to different regions in the world. As a consequence, the common bean presents adaptation to diverse environmental conditions. In the last two decades, *P. vulgaris* has been the target for multiple genomic studies, ending with the development of genetic maps, the identification of multiple quantitative traits loci, and the identification and use of molecular markers that co-segregate with genes conferring resistance to pests and diseases that affect the crop.

Desirable characteristics have been searched for within germplasm banks, with considerable success. For self-pollinated crops, such as common bean, the breeding methods most commonly used are bulk, pedigree, back-cross, and their modifications. In general, interspecific hybridization and exploration of genetic variability is very limited within the genus *Phaseolus*. In addition, some barriers to hybridization and genetic recombination have been observed within *P. vulgaris*, with great consequences for genetic and breeding studies (Aragão et al. 2008; Gepts et al. 2008). Another fact, which has to be considered, is that both inter- and intraspecific hybridization produce hybrid plants with undesirable characteristics. Thus, an extra period in the breeding program is necessary to obtain a commercial cultivar. Despite this drawback, the utilization of sexual hybridization has resulted in several important examples of gene transfer, on which many of our current cultivated cultivars have been based. In the field of genetic engineering, substantial scientific advances have been achieved with the crop in the last two decades. Genetic transformation techniques have overcome the former limitation of the introduction of foreign genes into bean genome. For instance, transgenic plants of *P. vulgaris* with resistance to the Bean golden mosaic virus, an important crop disease, have been tested in field conditions in Brazil (Aragão and Faria 2009).

11.2 Taxonomy and Natural Distribution of *Phaseolus*

The *Phaseolus* genus is placed within the subtribe Phaseolinae, the tribe Phaseoleae, the subfamily Faboideae (=Papilionoideae), and the family Fabaceae (=Leguminosae) (Freytag and Debouck 2002; Judd et al. 2008). According to Freytag and Debouck (2002), more than 400 species within the genus have been described since the 1700s, and this diversity remains to be uncovered in more details. The exact

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number of *Phaseolus* species currently accepted is not known, and a reasonable estimate would be 50–60 species (Debouck 2000). Only five species have been domesticated within the *Phaseolus* genus, in pre-Columbian times: *P. vulgaris* (common bean), *P. polyanthus* (year-long bean), *P. coccineus* (scarlet runner bean), *P. acutifolius* (tepary bean), and *P. lunatus* (lima bean).

The occurrence of *P. vulgaris* species in its wild form was described in Mexico, in most of the countries of Central America [Costa Rica, El Salvador, Guatemala, Honduras, and Nicaragua (Freytag and Debouck 2002)] and in the western countries of South America [Ecuador, Colombia, Peru, Venezuela (Debouck et al. 1993), Bolivia (Freyre et al. 1996), and Argentina (Von Burkart and Brücher 1953)]. Pictures of wild *P. vulgaris* (plants and seeds) are presented in Fig. 11.1.

According to Debouck et al. (1993), one of the main ecological features of the habitat for the species colonization is the pattern of rainfall distribution, with a rainy season for the plant growth until the blooming period or the beginning of the pod filling period, followed by a dry season period, when the plant maturation would take place. Maturation during the rainy season or in areas without dry season would lead to a significant reduction in fitness, due to seed germination inside the unopened pods. Debouck et al. (1993) described the ecological distribution of *P. vulgaris* in northwestern South



Fig. 11.1 Wild and cultivated *Phaseolus vulgaris*. (**a**) Plants of wild *P. vulgaris* showing the vigorous climbing nature, the large load of small pods, and the heliophilic nature of the species; photo by P. Gepts. (**b**) The first wild population of *P. vulgaris* collected in Ecuador (DGD-2762); it has been shown linked to the original branch of the species; photo by D. Debouck. (**c**) Plant of wild *P. vulgaris* collected in Colombia; photo by D. Debouck. (**d**) Flowers of wild *P. vulgaris*; photo by P. Gepts. (**e**) The small seeds are from *P. vulgaris* wild type and the large seeds are the result of hybridization between wild and domesticated black-seeded common bean planted by farmers; photo by P. Gepts. (**f**) Seeds, pods, leaves, and flowers of cultivated *P. vulgaris*. Seeds and pods are from different varieties; photo collection of Embrapa Arroz e Feijão

America (Venezuela, Colombia, Ecuador, and Peru). The wild populations of common bean described in the northwestern South America were collected in the Andes Mountain range, and the main ecological habitat was described as (semi-) dry montane tropical forests. As for the wild populations of *P. vulgaris* collected in Central and North America, the same ecological pattern of rainfall distribution seems to hold: fairly constant rainfall for early growth followed by an abrupt cease of rainfall (dry season initiates) during or shortly after the plant flowering period (Delgado-Salinas et al. 1988; Freytag and Debouck 2002).

The wild *P. vulgaris* was usually found in habitats with intermediary to high altitudes, such as pre-montane environments. Freytag and Debouck (2002) reported altitudes ranging from 800 to 2,000 m for the collecting sites of wild *P. vulgaris* from Mexico and Central America. As for the wild types from South America, Debouck et al. (1993) reported ranges of 960–2,090 m for Colombia, Ecuador, and Peru, whereas Freyre et al. (1996) reported ranges of 920–2,780 m.

In their review about the taxonomy and distribution of *Phaseolus* species in North and Central America, Freytag and Debouck (2002) mentioned that there were relatively few wild collections of *P. vulgaris* from Central America. The authors recalled the fact that early collectors (until the 1940s) did not find any wild types of the species in Mexico or Guatemala, contributing to an erroneous conclusion about the origin of the cultivated common bean. According to these authors, the Central American area would be on the edge of the distribution of wild *P. vulgaris*.

11.3 Relationships of *P. vulgaris* with Other *Phaseolus* Species

Many studies have tried to evaluate the genetic relationships of *P. vulgaris* with other *Phaseolus* species. These studies have taken into account different aspects such as morphological and phytogeographic reviews, molecular phylogenetic analyses, and reports on hybridization between the species. In a detailed review of herbaria and field collections, Freytag and Debouck (2002) recognized 15 sections within the *Phaseolus* genus, based on morphological differences of previously recognized species. *P. vulgaris* was placed within the Phaseoli section, along with *P. costaricensis*, *P. dumosus* (=*polyanthus*), and *P. albescens*. Molecular phylogenetics studies within the genus confirmed, in part, this assignment of species within the same section as *P. vulgaris*. Using internal transcribed spacer DNA sequencing combined with non-molecular data, Delgado-Salinas et al. (1999) performed a molecular phylogenetic analysis sampling around 50 *Phaseolus* species. Their results identified nine monophyletic clades within the *Phaseolus* genus, and the *P. vulgaris* clade included all the four species placed in the Phaseoli section (Freytag and Debouck 2002), in addition to *P. coccineus* and *P. acutifolius*. Other studies using molecular analyses showed some differences in the relationship between *Phaseolus* species (for example, Fofana et al. 1999), probably due to differences on sampling strategies (numbers of accessions and taxa examined) (Freytag and Debouck 2002), as well as differences for the type of DNA analyzed (Broughton et al. 2003).

According to Freytag and Debouck (2002), *P vulgaris* is fairly easily hybridized with *P. dumosus* (=*polyanthus*). As for the potential hybridization between *P. coccineus* and *P. vulgaris*, Broughton et al. (2003) recalled that these species were frequently intercrossed from 1940 to 1985, and they claimed that *P. coccineus* would hold the greatest potential as genetic diversity resource once the primary gene pool of *P. vulgaris* has been completely exploited. However, Freytag and Debouck (2002) contradict the close relationship between these two species, emphasizing that although they have been crossed in multiple occasions, they are rarely found growing together, and little fertile and useful progeny have yet been obtained. Many studies have also tested the hybridization between *P. vulgaris* and *P. acutifolius*, and less compatibility between these species was reported (Freytag and Debouck 2002; Broughton et al. 2003). Other species have been crossed with *P. vulgaris*: *P. angustissimus*, *P. filiformis*, *P. maculatus*, *P. ritensis* (reviewed in Freytag and Debouck 2002), *P. parvifolius*, and *P. lunatus* (reviewed in Broughton et al. 2003), but no fertile hybrids have been obtained.

11.4 Domestication: Two Gene Pools

Common bean is a typical example of a non-centric crop (Harlan 1992), since domestication of *P. vulgaris* occurred over a wide area. The species has been

domesticated independently in Mesoamerica and the southern Andes, based on several kinds of data (distribution of wild populations, archeological remains, historical texts, and evolutionary studies based on several types of molecular markers), including phaseolin (the major seed protein in *P. vulgaris*) (reviewed in Gepts 1998; Gepts et al. 2008; McClean et al. 2008; Kwak et al. 2009). To discuss the evolution of common bean, Gepts (1998) defined Mesoamerica as the region that encompasses the southern half of Mexico and the northern half of Central America, while the Andean region was defined as the one that encompasses southern Peru, Bolivia, and Argentina. A wild common bean from northern Peru was identified as a presumed ancestral gene pool of the species (Debouck et al. 1993; Kami et al. 1995). These wild beans probably dispersed both northwards and southwards to form two distinct gene pools before domestication (reviewed in Gepts 1998; Broughton et al. 2003; McClean et al. 2008). The precise locations of common bean domestication within each one of these two major regions (Mesoamerica and Andes) are still under debate. Kwak et al. (2009) recently proposed a region in West-central Mexico, in the Rio Lerma–Rio Grande de Santiago basin, as the putative Mesoamerican domestication center of common bean, based on molecular analysis using microsatellites.

As a result of this bicentric process of domestication, the two gene pools are usually very clearly distinguished in most (if not all) of the studies assessing the genetic diversity of common bean collections, either by different kinds of molecular data (Gepts 1988; Koenig and Gepts 1989; Emydgio et al. 2003; Pallottini et al. 2004; Kwak et al. 2009) or by morphological characters (Singh et al. 1991).

These two gene pools are also separated by partial reproductive isolation, both in wild and domesticated populations (Gepts and Bliss 1985; Koinange and Gepts 1992), which leads to hybrid weakness in the F_1 (Gepts and Bliss 1985) and later generations (Singh and Molina 1996). According to Gepts (1998), the existence of this reproductive isolation and the degree of divergence at the molecular level suggest that these two gene pools would actually represent two subspecies.

This strong subdivision of common bean domesticated gene pool into two major groups presents important implications for the use of this genetic resource. First, host–microbe co-evolution has been suggested in the case of common bean, for different pathogens (Guzmán et al. 1995; Geffroy et al. 1999; Araya et al. 2004; Kelly and Vallejo 2004; Mkandawire et al. 2004) as well as for *Rhizobium* (Aguilar et al. 2004). Physiological traits related to photosynthesis may also distinguish these two gene pools (Lynch et al. 1992; Gonzalez et al. 1995).

Second, limitations related to reproductive barriers between accessions from the two different gene pools are also important. Broughton et al. (2003) referred a variety of studies that found it difficult to obtain high-yielding genotypes in Andean × Mesoamerican crosses because of outbreeding depression. However, as Gepts (1998) claims, assessing the diversity from both common bean gene pools may also constitute an opportunity, since recombination between these two pools could provide new and interesting gene combinations, if the reproductive isolation could be overcome.

Besides the main division of common bean gene pool into the Andean and the Mesoamerican groups, a further genetic classification of common bean into ecogeogaphic races was also proposed (Singh et al. 1991). For this classification into races, Singh et al. (1991) considered a multiple set of characteristics such as molecular and morphological characterization, agronomic traits, reproductive isolations, ecological adaptation, and geographical distribution. Six races of common bean were defined: races Durango, Jalisco, and Mesoamerica are from the Mesoamerican gene pool, whereas races Chile, Nueva Granada, and Peru are from the Andean gene pool. The races' classification have been often used and discussed in studies of genetic diversity of common bean, particularly by the group of bean scientists from the Centro Internacional de Agricultura Tropical (Beebe et al. 2000; Blair et al. 2006a, 2007, 2009a; Díaz and Blair 2006).

11.4.1 Domestication Syndrome and Diversity Reduction

The phenotypic differences that cultivated plants often show if compared with their wild-growing progenitors (Harlan 1992) have been named as domestication syndrome (Koinange et al. 1996). The two most important attributes of the domestication syndrome in common bean are the loss of dispersal ability and seed dormancy (Koinange et al. 1996).

Other important traits that present changes with domestication in the species are the ones related to growth habit. While the wild types of common bean often sprawl or climb, the cultivated common bean usually presents determinacy, non-twining branches, few vegetative nodes, and long internodes. As a result, the cultivated common bean types usually present a more compact growth habit if compared with the wild progenitor.

Another important trait related to the domestication syndrome in common bean is the life cycle. According to Koinange et al. (1996), determinacy (the early transition from a vegetative terminal meristem to a reproductive one) has, by its very nature, an effect on shortening the plant life cycle. Freytag and Debouck (2002) described wild *P. vulgaris* as a perennial plant, tending to be annual in many wild populations. As for the cultivated types, almost all of them are annual plants (Freytag and Debouck 2002).

The study of Koinange et al. (1996) was the first one to investigate, directly, the inheritance of the domestication syndrome in a legume crop. The following traits were confirmed, in this study, as related to this syndrome in the species: growth habit, phenology, seed dispersal, dormancy, and size of fruit and seed. An interesting finding of this study was the fact that the domestication genes identified in the species appeared to be concentrated in just three genomic regions.

Many crops are marked with a reduction of diversity during and after domestication (Doebley 1989; Gepts 1998). For common bean, this aspect also holds true, and the reduction of diversity associated with the domestication process was documented in this crop more than 15 years ago, based on DNA molecular analysis (Sonnante et al. 1994).

11.4.2 The Domesticated Pool Occupied New Regions

The process of dissemination of common bean from its domestication centers to other regions in the World, where it is currently cultivated, is not extensively studied, and the subject remains to be evaluated in more depth. In a classic study about the origin, dispersal and variability of *P. lunatus* (the lima bean, one of the four cultivated *Phaseolus* species) in the American continent, Mackie (1943) discussed three routes of dispersal of this crop from Central America, in pre-Columbian times: the Hopi, the Carib, and the Inca routes. Most of these routes were also trade routes, and they dispersed also other crops, the common bean among them. The Hopi route, for example, connected Central America northward to Arizona State, in the United States, and was responsible for the dispersion of common bean, lima bean, tepary bean (*P. acutifolius*), corn, and squashes. The Carib route would connect Central America continent to the Caribbean islands, Cuba, the former West Indies, and the Antilles Islands, reaching Brazil. The Inca route dispersed the beans southward to the Andean region, in South America.

Gepts and collaborators discussed the dissemination pathways of common bean to Africa, Europe, and other American regions based on phaseolin (a major seed protein) variability. Gepts et al. (1988) postulated that common bean had been introduced in Brazil at least from two routes, one (or more than one) for the Mesoamerican types and another(s) one for the Andean types, since both types of common bean occurred in Brazil. For the routes that could have dispersed Mesoamerican common bean types into Brazil, Gepts et al. (1988) postulated two options: from Mexico, following the Caribbean Coast into Colombia and Venezuela and eventually into Brazil, and from Mexico for the Caribbean islands and from there to Venezuela, Colombia, and Brazil. This last route postulated by Gepts et al. (1988) was similar, but not identical, to the Carib route described by Mackie (1943). As for the possible routes that dispersed the Andean common bean types into Brazil, Gepts et al. (1988) suggested also two options: a route from Colombia and Ecuador, and a post-Columbian introduction from immigrants that came from Europe, where the Andean common bean types predominated (Gepts and Bliss 1988). Historical reports also indicated that beans were already cultivated by the Brazilian native populations when the Portuguese colonizers first arrived in Brazil (Léry 1576; Hoehne 1937). In addition, archeological samples from Brazil also supported the hypotheses of common bean cultivation in Brazil before the European conquest (Prous et al. 1984; Freitas 2006).

As for the dissemination of common bean to North America, Gepts et al. (1988) identified that most of the types cultivated in the southwestern United States presented a phaseolin type characteristic from the Mesoamerican gene pool. Historical and linguistic

records of common bean cultivation in that region before the European conquest, such as Native American names for the crop, as well as archeological records (Kaplan 1981), helped Gepts et al. (1988) postulating the hypothesis that common bean has been introduced in that part of the United States predominantly from the Mesoamerican region and in pre-colonization times [similarly as the Hopi route, postulated by Mackie (1943)]. According to Gepts et al. (1988), in the northeastern United States predominated the Andean common bean types, and the authors suggested two possible routes, both in poscolonization times: from Europe and directly from the Andean countries (Chile).

Gepts and Bliss (1988) identified that most of common bean cultivars in the Iberian Peninsula presented an Andean phaseolin type that had been described predominantly in Chile, pending on more investigation. As for the rest of Europe, the authors identified the predominance of cultivars with Andean phaseolin types that occurred also in other Andean regions. More recently, other studies have confirmed the predominance of Andean types among the European common bean (Logozzo et al. 2007; Marotti et al. 2007).

The results of Gepts and Bliss (1988) provided a new outlook on the origin of common bean in the African continent, where most of the accessions sampled presented also an Andean phaseolin type. Previous hypothesis postulated that common bean had been introduced in Africa from Brazil, associated to the slaves' trades (Evans 1976). However, the Mesoamerican common bean types predominated in Brazil (Gepts et al. 1988). According to Gepts and Bliss (1988), differential adaptations among cultivars of different origins and differences on consumption preferences may also explain the maintenance (or not) of the different common bean types in the regions where they have been introduced after domestication.

Zhang et al. (2008) claimed that China could be another secondary center of diversity for common bean, where the crop has been introduced around 400 years ago. Although the majority of the landraces cultivated in China were classified as Mesoamerican types, the authors postulated that a higher number of introductions of the species in this country were from Andean types, since the study detected a higher diversity within the Andean group of Chinese landraces, when compared with the Mesoamerican group.

As the domesticated common bean was spread from the specific habitats within its primary center of diversification (subhumid pre-montane forests of Central America and western South America) to different regions and habitats within the World, some other aspects/traits of the species were also affected. One of the physiological traits affected in the species during this process is the sensitivity to photoperiod. The dissemination of cultivated common bean from the species' domestication centers (in the tropics) to areas with higher latitudes has resulted in selection for genotypes insensitive to day length, if compared with the wild types (Koinange et al. 1996; Broughton et al. 2003).

Differences on the adaptation of the species to stressing soil characteristics after domestication constitutes a subject that has not been extensively investigated and debated, although very relevant. The domesticated gene pool of common bean seems to harbor more genotypes adapted to acid and low fertility soils, if compared to the wild gene pool in the species (Beebe et al. 1997). Toro et al. (1990) pointed out that wild *P. vulgaris* was typically found in a subhumid pre-montane forest environment in which soils are less weathered than in more humid areas of the tropics. Freytag and Debouck (2002) described the soils of the areas where wild *P. vulgaris* was collected in Mexico and Central America as mineral or volcanic types, usually derived from limestone (high fertility), schist, granite, lava (high fertility), or tuffa (high fertility). In contrast, secondary centers of common bean diversity, such as Brazil and Africa, present older and more weathered soils, with more acidity, with higher contents of toxic aluminum, and lower availability of nutrients, phosphorus among them (Goedert 1986; ISRIC 2010).

Adaptation for cultivation in lower altitudes (therefore, adaptation to warmer temperatures) is another aspect that has probably been affected in the species after the domesticated gene pool moved to other regions. While the wild *P. vulgaris* was usually found in pre-montane habitats, the domesticated common bean occupied, for example, the lowland South American regions (Brazil, Venezuela, and northern Colombia) (Gepts et al. 1988). Burle (2008) estimated altitudes ranging from 0 to 1,200 m for the collecting sites where common bean landraces have been cultivated in Brazil.

11.5 Genetic Analyses

The genetic variability of cultivated common bean varieties (*P. vulgaris*) is thought to be smaller than that of wild common bean due to a genetic bottleneck and founder effect that occurred during crop domestication, which reduced the possibilities of genetic gain on breeding programs (Gepts 1988). Since wild progenitors are the foundation of landraces and landraces are the foundation of modern cultivars, elucidation and understanding the structure of genetic diversity in the gene pool of common bean are essential for broadening the genetic base of cultivars to sustain the breeding programs (Singh 2001; Chacón et al. 2005). Studies on the domestication and organization processes of the common bean genetic variability were first based on morphological traits and biochemical markers. In the late 1980s, following the PCR methodology inception, a number of molecular marker technologies have been developed. In the 1990s, with the emerging advances of the DNA techniques, associated to the reduction of the costs and greater accessibility to the molecular approaches, the number of available molecular markers became limitless, making possible the investigation of different aspects of the genetic structure of crops species and accessible the construction of more informative and representative genetic maps. The characterization of genetic diversity among common bean accessions from divergent gene pools using molecular markers has been shown to be effective to elucidate mechanisms of origin and evolution, to investigate the effects of domestication processes in the reduction of the genetic diversity that has characterized common bean gene pools, and to allow an efficient management and effective exploitation of the germplasm (Gepts 2004). On the basis of diverse classes of molecular markers, which explore genome-wide variation, studies of genetic structure between and within gene pools of a collection of wild *P. vulgaris* has shown that wild accessions of *P. vulgaris* clearly clustered into Andean, Mesoamerican, and intermediate gene pools (Freyre et al. 1996). Several investigations have shown the *P. vulgaris* diversity and most likely region of origin from its gene pool (Kwak et al. 2009; Blair et al. 2010), the level of genetic diversity and origin of Andean cultivated and wild bean (Beebe et al. 2001), the level and direction of gene flow between wild and domesticated bean populations (Papa and Gepts 2003; Papa et al. 2005), and the analysis of variability that allowed to conclude about the high levels of variability both within wild bean populations and landraces, where the wild samples grouped according to their geographic distribution and the landraces based on their seed morphobotanical descriptors (Galván et al. 2010).

As an advantage of the genetic divergence, wild common beans represent an important reservoir of alleles and can, therefore, be used as an additional source of genetic variability in common beans-breeding programs, since they cross easily with the modern *P. vulgaris* cultivars, producing fertile generations as demonstrated by Singh (2001). In addition, the use of wild genitors overcome the reduced polymorphism attributed to the narrow genetic base observed among cultivated common bean (Maciel et al. 2003), mainly among crosses within the same gene pool as reported by Frei et al. (2005) and Blair et al. (2006a). More recently, Pérez-Vega et al. (2009) characterized a group of landraces collected in Spain and described a considerable diversity among them, suggesting as an important secondary center of genetic diversity of common bean that must be preserved in order to maintain part of the genetic diversity of the primary centers.

Acosta-Gallegos et al. (2007) in a review described the use of the genetic diversity from wild germplasm of common beans in the pre-breeding programs as a valuable means to recover the lost of diversity through the search of potentially favorable alleles in wild ancestors, aiming at the development of new cultivars with a higher genetic diversity and more resistance to biotic and abiotic stresses. Afterwards, important linkage maps were developed that allowed significant advances in the knowledge regarding the structure and organization of the *P. vulgaris* genome. The first genetic map for common beans, which integrated a diverse class of molecular markers, was nominated "Florida map," and was based on restriction fragment length polymorphism (RFLPs), isozyme, and random amplified polymorphic DNA (RAPD) markers and phenotypical traits (Vallejos et al. 1992). Later on, Nodari et al. (1993a, b) developed linkage maps derived from the crossing between the line BAT93 and the cultivar Jalo EEP558, nominated as "BJ" population, embracing a large set of RFLP and RAPD markers. In a following study, Adam-Blondon et al. (1994) constructed the "Paris map," also based on RFLP and RAPD markers. In order to align these

maps, a core linkage map was established in the recombinant inbred BJ population with a total length of 1,226 cm and it included a large set of markers (Freyre et al. 1998), followed by the assignment of the first set of codominant microsatellite markers into a *P. vulgaris* molecular linkage map (Yu et al. 2000). Subsequently, a large and new set of microsatellite markers begun to be integrated into common beans' linkage maps (Blair et al. 2003, 2006a), resulting in a map based exclusively on microsatellite markers mapped in the BJ population (Grisi et al. 2007). The correspondence between the linkage groups and the chromosomes was established as described by Pedrosa et al. (2003) and adapted by Grisi et al. (2007). Recently, a new expanded version of the core linkage map also using the BJ population was released, which included markers with putative gene function (Hanai et al. 2009). Currently, single nucleotide polymorphism (SNP)-based genetic markers are being used to create dense genetic linkage maps. In addition, SNPs can provide gene-based markers to identify candidate genes associated with quantitative trait loci (QTL) (Rafalski 2002). The first report of SNP mapping in *P. vulgaris* was described by Galeano et al. (2009), which placed a total of 118 new marker loci into an integrated molecular map.

Following the construction of linkage maps, genome regions involved in the control of simple as well as complex heritable traits are being identified. QTLs for important traits have been located in the genus *Phaseolus* using a variety of molecular marker classes and several types of segregating populations. The experimental approaches used have been effective to identify genomic regions that have a significant effect on the expression of QTLs, providing information about the number and the magnitude of the effects of the genes controlling proportions of the total variation, subjected to a pronounced environmental effect and dependent on the genetic background. Most of the target traits are related to agronomically relevant measures for the breeding programs and can be considered as useful traits in the selection process to identify lines with desirable phenotypes contributing to the increase in the efficiency of the common bean breeding programs. QTLs for complex traits have been identified from wild relatives and landraces of common beans since they provide a large variation of naturally occurring alleles for QTL mapping and are used for crop improvement. Koinange et al. (1996) were the first to map QTLs using a recombinant inbred population resulting from a cross between a wild and a cultivated common bean employing molecular markers. In this report, traits related to the domestication syndrome in common bean were evaluated, and QTLs with large effects (>25–30%) related to growth habit and phenology, seed dispersal and dormancy, size of fruit and seed concentrated in three regions of the genome accounting for substantial component of the phenotypic variance (>40–50%) were detected. In subsequent studies, QTLs related to yield including days to flowering, days to maturity, seed weight, number of seeds per pod, and architectural traits were identified through advanced backcross QTL analysis, in which QTLs for seed size showed positive alleles from the wild parent (Blair et al. 2006b). More recently, traits related to growth habit, such as plant height, internode length, and number of branches per plant, have being analyzed by QTL mapping (Checa and Blair 2008). Among the factors that affect crop yield, root traits play a major role due to the possibility to increase the efficiency to extract and absorb water and nutrients from the environment. QTL analysis of root architecture traits has been conducted that allowed the establishment of correlation with mineral soil tolerance. Beebe et al. (2006) were the first to conduct such studies, followed by Ochoa et al. (2006), who mapped adventitious root formation QTLs under contrasting soil phosphorus concentrations. More recently, a study locating QTLs for root architecture traits under low phosphorus tolerance in an Andean bean population was described (Cichy et al. 2009a).

Several studies have described QTLs controlling different traits as being mapped in the same chromosomal regions. However, if these analyses are based on low-resolution linkage maps, it will not be possible to determine whether the traits are controlled by single genetic elements that exhibit pleiotropic effects, or they contain several tightly linked genes. QTL overlapping has been extensively described, for example, QTLs for increased concentrations of different seed micronutrients (Fe and Zn) that co-localized showing a positive correlation without environment interaction reported in an Andean bean population (Cichy et al. 2009b). Only fine-mapping on the target genomic regions has been of help to address these questions (Hanemann et al. 2009). However, regarding all experimental QTL researches that have been conducted along the years, only a few QTLs related to the same traits has been mapped at common locations in the

linkage maps. This is in part due to the different mapping populations used, the interactions related to different genetic backgrounds of the genotypes, the environmental effect on the trait, the limitations due to the incomplete alignment of linkage groups between the studies, and the reduced level of genetic information content of the markers used for the linkage analyses. The increasing use of codominant markers widely distributed across the genome and their potential to be transferable between different genotypes, as microsatellite markers, depicts an amenable perspective for the establishment of consolidated linkage maps, and making the QTL mapping analyses useful among different research groups. Currently, these studies have provided insights into the genetic control of several agronomically important traits in common beans and strongly represent an initial step toward marker-assisted selection for complex traits.

The advanced genomic studies of common beans can be successfully accelerated due to the development of expressed sequence tag (EST) database, genome sequencing, bacterial artificial chromosome libraries, and physical mapping. As in February 2010, there were 83,847 entries of ESTs in the National Center for Biotechnology Information database. These sequences, when submitted to functional annotation studies, provide a valuable resource for the development of markers based on candidate gene approach, facilitating the direct establishment of probable relationships between these and specific QTLs through the development of a transcriptional map. Over the last 5 years, EST sequencing projects were initiated in common beans, contributing until now with an increase of 46 times in the number of EST sequences publicly available. Microsatellites or simple sequence repeats (SSR) are the most important and popular molecular markers that can be developed from ESTs. Approaches of mining for repetitive sequences in EST databases have been successfully applied for common beans (Blair et al. 2003, 2009b; Hanai et al. 2007). As part of the genomic resources available for common beans, Gaitán-Solís et al. (2008) reported identification of a high frequency of SNPs through the genomic DNA when analyzing a set of cultivated and wild bean genotypes belonging to the Mesoamerican and Andean gene pools, suggesting that diverse *P. vulgaris* genotypes could be useful for SNP identification. An initiative toward a whole structural genome sequencing of common bean is currently underway by an international collaborative project including Mexico, Brazil, Spain, and Argentina, funded by the Ibero-American Development Program for Science (CyTed). The *P. vulgaris* sequencing genome information is a promising field with high potential to provide significant advances in common beans' genomics, useful for geneticists, breeders, and genetic resource specialists.

11.6 Tissue Culture and Genetic Engineering

Plant regeneration in the genus *Pheseolus* has been achieved for several species through de novo organogenesis, somatic embryogenesis, and proliferation of shoot meristems from areas surrounding a shoot bud. Numerous attempts have been made to regenerate *P. vulgaris* plants from several types of isolated cells and tissues. Although no satisfactory results have been achieved, some methodologies have described shoot organogenesis (through multiple shoot induction) of the apical and axillary meristems from bean embryonic axis (McClean and Grafton 1989; Malik and Saxena 1992; Mohamed et al. 1992, 1993; Aragão et al. 1996; Aragão and Rech 1997). Cruz de Carvalho et al. (2000) employed the transverse thin cell layer (tTCL) method to optimize the frequency of shoot regeneration without an intermediate callus stage. Recently, organogenesis protocols have been developed or optimized for several genotypes (Delgado-Sánchez et al. 2006; Mohamed et al. 2006; Arellano et al. 2009; Kwapata et al. 2010).

Zambre et al. (2001) described regeneration of several genotypes of *P. polyanthus* combining thidiazuron (TDZ) and indole-3-acetic acid (AIA) to induce morphogenic green nodular callus. Regenerated shoots that formed roots in vitro (40%) were established in the greenhouse, whereas non-rooted shoots could be established in vitro by grafting performed on the hypocotyl of *P. vulgaris* seedling rootstocks. Somatic embryogenesis and subsequent plant development have been reported for *P. coccineus* and *P. acutifolius* (Kumar et al. 1988; Genga and Allavena 1991).

Recently, the first protocol for callus induction and shoot regeneration has been reported for *P. lunatus* (Kanchiswamy and Maffei 2008). Combining thidiazuron and IAA for the induction of callus followed by

BAP for the induction of shoots, it was possible to regenerate plants with fastest response and with the highest percentage of shoot regeneration (40–60%) from epicotyls (Kanchiswamy and Maffei 2008).

To date, *Phaseolus* species have been transformed genetically with limited success, with most of the efforts being concentrated on *P. vulgaris* (Aragão et al. 2008) and *P. acutifolius* (Dillen et al. 1997; Clercq et al. 2002). *P. acutifolius* can be used as a "bridging" species to introduce transgenes into the economically more important species *P. vulgaris*.

P. vulgaris has been successfully transformed using both *Agrobacterium* and biolistic-mediated systems (for a review see Nagl et al. 1997; Liu et al. 2005; Aragão et al. 2008). These systems have been used to introduce several useful traits into common bean plants, and the first commercial lines are now under development in Brazil (Aragão et al. 2008; Aragão and Faria 2009).

Dillen et al. (1997) have described a methodology to transform *P. acutifolius* (genotype NI 576) utilizing *Agrobacterium tumefaciens*. Regenerating calli, obtained from bud explants, were co-cultivated with *A. tumefaciens* C58C1Rif^R (pMP90) harboring a binary vector with the neomycin phosphotransferase II (*npt*II) and β-glucuronidase (*uid*A) marker genes. Using this system, transgenic plants containing a genomic fragment encoding the *P. vulgaris* arcelin-5a protein were obtained. The *P. acutifolius* transformation system was further improved with an *A. tumefaciens* strain carrying nopaline-type virulence genes and when calli were infected with *Agrobacterium* cells in the early-log growth phase. With these modifications, an efficient and reproducible transformation procedure was established for the *P. acutifolius* genotype NI576 (Clercq et al. 2002). Another *Agrobacterium*-mediated method was further developed by Zambre et al. (2005) allowing the generation of morphologically normal transgenic plants of *P. acutifolius*. However, the shoots did not root in approximately one-third of the lines. The non-rooted shoots had to be grafted and in vitro establishment rate for the grafts was 80–100%. All the plants regenerated with this procedure were stably transformed, and the introduced foreign genes (*uidA*, *nptII,* *arc1* and *acr5*) were inherited in Mendelian fashion in most of the transformants. In addition, plants expressing arcelin genes (Arcelin 5 and Arcelin 1) were tested for resistance to *Z. subfasciatus* (Mexican bean weevil) and the results showed a delay in the emergence of adult insects. However, due the fact that the differences were small, the expression of *arc1* or *arc5* transgenes was considered not to influence insect biology (Zambres et al. 2005)

11.7 Concluding Remarks

Important information has been obtained about the diversity of *Phaseolus*, particularly in the last three decades. At the same time, many interesting questions have been raised about the species' diversity. The regions of natural distribution of the species have been described, in the Americas, making it possible to understand the main ecological features for the wild species' growth. The relationships of *P. vulgaris* with other species within the genus have also been investigated, from multiple perspectives. Some five species have been pointed as closely related to *P. vulgaris*, despite some on-going debate on the subject. The strong genetic structure of the species, organized into two major gene pools (the Andean and the Mesoamerican), is unequivocal in the common bean scientific community and that presents important implications for the use of the genetic resources. The history of common bean dispersion from its center of domestication to other regions in the world has been discussed in the literature, although it remains to be investigated in more depth. As the domesticated *P. vulgaris* occupied other regions in the world, important traits related to the adaptation of the species to environmental stresses were affected. This is another important aspect that deserves further investigation, and is very relevant for the use of the crop genetic resources.

An increased understanding of common bean genome associated with contributions from biotechnology will provide an opportunity for breeders to accelerate the development of new varieties with valuable agricultural traits. Common bean has a relatively small genome with 11 haploid chromosomes and genomics studies are in progress. These will facilitate an excellent scenario for the introduction of useful traits as well as studding gene function in *P. vulgaris* plants. There is considerable interest in the introduction of genes for several useful traits in common bean such as resistance against virus, insect, bacteria, and fungi, environmental stress tolerance, and improvement in nutritional properties. In addition, manipulation of

plant architecture and phenological characteristics might facilitate management, increasing yield, quality, and diversity. Although regeneration systems have been developed for several *Pheseolus* species, transformation has been achieved with limited success. From a basic and applied genetics and molecular biology perspective, it would be interesting if it would be possible to transform most *Phaseolus* species, facilitating studies on gene expression and comparative genomics. Several groups are now making efforts for introducing useful traits into *P. vulgaris* and *P. acutifolius* by genetic engineering, mainly in Brazil, United States, Colombia, and Mexico.

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Chapter 12
Pisum

T.H.N. Ellis

12.1 Introduction

Pisum has long been recognized as a significant genus; the Papilionoid legumes as a whole are often typified as having pea or pea-like flowers (Lewis et al. 2005). Pea (*Pisum sativum* L.) is the original model organism for genetic studies. It was first used in this way by Knight (1799) to show that male and female individuals contributed equally to the progeny of a cross. Olby (1985) points out that Mendel (1866) seemed to be impressed by this choice, for he gives the same reasons, in the same order as Knight, for choosing to study pea. *Pisum* has been a workhorse for plant biochemistry but has declined in its use as a model species for genetics largely as a consequence of its large genome size (Macas et al. 2007), small number of seeds set per cross, and its recalcitrance in tissue culture that makes transformation difficult (Schroeder et al. 1993; Bean et al. 1997). Nevertheless, just preceding the ascendancy of the model system *Arabidopsis thaliana*, working solely from knowledge of the underlying biochemistry and genetics, it was possible to identify Mendel's wrinkled-seed gene, *r* (Bhattacharyya et al. 1990).

Although we have seen a period of relative decline in the study of *Pisum*, a number of recent developments hold promise for a resurgence. We now have a systematic mutant population for reverse genetics (Dalmais et al. 2008), bacterial artificial chromosome libraries (Coyne et al. 2007; and see Hofer et al. 2009), and an effective gene silencing system (Constantin et al. 2004) as basic resources. Together with the availability of high-throughput sequencing and genotyping methodologies, these resources hold promise for rekindling interest in basic genetic studies in pea.

It is clear that pea has been an object of study for many years; however, much of the revolution in molecular genetics, especially genomics-based approaches, have yet to have their impact. Nevertheless, several marker-based studies have attempted to describe the pea genome and the pattern of allelic diversity within the genus. This chapter focuses on what has been learnt about the relationship between pea and its wild relatives and the attendant attempts to understand how these resources can be accessed and deployed.

12.2 Taxonomy and Geography

Pisum is a genus within the Fabeae (formerly Vicieae) closely related to *Lathyrus*, with this pair of genera closely related to *Vicia* and *Lens*. *Cicer* is an outgroup to these and the Trifolieae (Wojciechowski 2003; Fig. 12.1). The close relationship between *Pisum* and *Lathyrus* has been reflected by former versions of species names, such as *L. japonicus* Willd ssp. *maritimus*, which was previously called *P. maritimum* (see the International Legume Database and Information Service database, *http://www.ildis.org*). Similarly, the enigmatic *Vavilovia formosa* was formerly considered to be *P. formosum*.

Although the systematics of *Lathyrus* has been carefully revised (Kenicer et al. 2005; Kenicer 2006), the relationship between *Pisum* and *Lathyrus* is not completely clear. These studies used a single representative of *P. sativum*, and the authors note that the support for the separation of *Pisum* and *Lathyrus* is weak. Kenicer et al. (2005) placed *Pisum* close to

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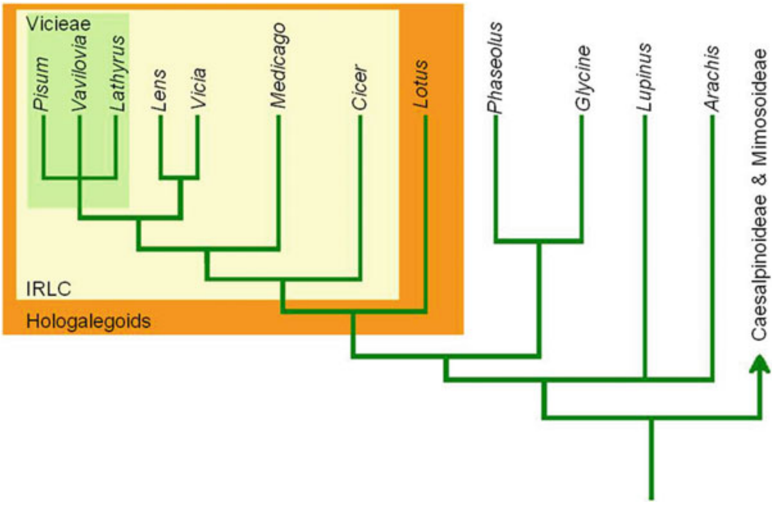


Fig. 12.1 Taxonomic relationships of *Pisum*. A summary phylogenetic tree based on the references in the text is presented. The family Vicieae (or Fabeae see Lewis et al. 2005) is highlighted in *gray*. The Inverted Repeat Lacking Clade (IRLC, where the chloroplast DNA lacks the usual inverted repeat organization) is highlighted in *light gray*. The hologalegoids are highlighted in *dark gray*

L. nissolia (grass pea), which, as the common name suggests, has a very unusual leaf form. It is, therefore, interesting to note that a distinguishing feature of *Pisum* vs. *Lathyrus* is the way in which leaflets open (*Pisum* like a book vs. *Lathyrus* unrolling).

For convenience, in this discussion, I consider *Pisum* as monophyletic, but this has not been established rigorously with modern taxonomic analytical methods and must, therefore, remain in some doubt.

Pisum has variously been considered species-rich or monospecific (discussed in Blixt 1972). The Kew database (*http://epic.kew.org*) lists 82 different species of *Pisum*, although not all names are "valid," according to the International Plant Names Index (*http://www.ipni.org*). In the USDA-GRIN database (*http://www.ars-grin.gov*), the names of 13 species are listed, 3 of which correspond to other genera, and of the remaining 10, "*arvense*" and "*commune*" are considered synonymous with "*sativum*." This leaves us with the names *P. abyssinicum*, *P. elatius*, *P. fulvum*, *P. humile*, *P. jomardii*, *P. sativum*, *P. syriacum*, and *P. transcaucasicum*. Several of these names also occur in the ILDIS database and in both some also appear as subspecies or varieties of *P. sativum*. The ILDIS names follow Maxted and Ambrose (2001) and recognize *P. sativum* ssp. *sativum*, *P. sativum* ssp. *elatius*, *P. abyssinicum*, and *P. fulvum*.

Despite these issues about the identity of taxa, the geographic distribution of *Pisum* is fairly clear. *Pisum* is an Old World genus with a broad distribution of non-cultivated types from the western end of the Mediterranean to the east of the Himalayas. *P. fulvum* is limited to the eastern end of the Mediterranean basin, whereas *P. elatius* is distributed all around the Mediterranean (in North Africa and southern Europe) and extends eastward south of the Caspian Sea and along the southern foothills of the Himalayas. *P. sativum* has a worldwide distribution and is grown in most temperate regions as well as in highlands of tropical areas. *P. abyssinicum*, as its name implies, has a much more restricted distribution corresponding to the highlands of Ethiopia (Westphal 1974) although some accessions have been attributed to Yemen.

12.3 Genetic Diversity Within *Pisum*

12.3.1 Marker Types and Diversity

A wide range of marker types is available for studying genetic diversity, and it is clear from previous studies that different markers have different utility. Lu et al. (1996) compared several marker methods and concluded that, of the several marker types tested, only the intersimple sequence repeat PCR method was unreliable for the analysis of diversity in *Pisum*. Ellis et al. (1998) noted that amplified fragment length polymorphism (AFLP) and sequence-specific amplified polymorphism (SSAP) methods were generally in agreement, but AFLP exaggerated the differences among *P. abyssinicum* accessions. Knox and Ellis (2001) showed that DNA methylation is a likely

cause of this exaggeration of the estimated genetic distance among *P. abyssinicum* accessions with respect to wild *Pisum*. Jing et al. (2007) used single-nucleotide polymorphisms to estimate genetic distances in *Pisum* and showed that these agreed well with estimates from retrotransposon insertion site variation.

Microsatellites or simple sequence repeats (SSRs) are popular markers, and they are highly polymorphic. They give excellent discrimination between closely related individuals, and are sufficiently sensitive to detect novel length variants that arise in the sperm of a single human (Brinkmann et al. 1998). Microsatellites can have a mutation rate of the order of 10^{-4} per locus per meiosis in humans. Vigouroux et al. (2002) found a slightly slower rate of microsatellite length mutation in maize, whereas Raquin et al. (2008) reported a higher rate in wheat. Jing et al. (2005) estimated the rate of transposition of the *PDR1* retrotransposon in pea as $\sim 5 \times 10^{-7}$ and of silent nucleotide changes as $\sim 10^{-8}$ to 5×10^{-9}. These are three to four orders of magnitude slower than microsatellite mutation rates (assuming one generation per year). As *Pisum* is incredibly diverse by these measures (Jing et al. 2007), for microsatellite markers the risk of homoplasy in wide surveys of pea germplasm is high.

Homoplasy arises when alleles are identical, but not identical by descent. For example, an A to G transition may occur, and then later, in the population carrying the G allele, a G to A transition may occur. Now there are two indistinguishable alleles (carrying the A) that have different ancestry. This problem vexes taxonomists, but is unlikely to be a problem for intraspecific variation because of the slow nucleotide mutation rate. For microsatellites, the risk is relatively much higher because homoplasy will occur at the square of the ratio of the two mutation rates, that is, the risk is about six to eight orders of magnitude greater for microsatellites as for single-nucleotide polymorphisms. Whether homoplasy is actually a problem or not depends on the actual mutation rate and the time since the last common ancestor. Jing et al. (2007) estimated the age of alleles segregating in *Pisum*, and found this to be 1.9 ± 0.7 million years. If we take 10^{-4} as the microsatellite length mutation rate per year, then we expect on average 2% of (correct) microsatellite allele calls to misattribute ancestry. Obviously this will be a less severe problem in narrow germplasm and more severe in wide germplasm. This effect could be observed in a plot of genetic distance (D) vs. genetic distance measured by microsatellites. However, genetic distance is never known; we have only measures of genetic distance, but we should see a distortion, if we compare microsatellite measures of genetic distance to those from a process with a lower mutation rate. This is illustrated in Fig. 12.2 that clearly shows that microsatellites give larger estimates of genetic distance than other markers, especially for closely related individuals. This is expected because microsatellites are highly polymorphic, but at higher values of D, the microsatellite measure is relatively lower (the slope of the regression line is less) indicating the effect of homoplasy.

12.3.2 Accession Diversity

Several studies have used molecular markers to characterize the distribution of genetic diversity in *Pisum* (Ellis et al. 1998, 2005; Pearce et al. 2000; Ford et al. 2002; Vershinin et al. 2003; Baranger et al. 2004; Jing et al. 2005, 2007, 2010; Tar'an et al. 2005; Smýkal et al. 2008a, b; Zong et al. 2008, 2009) and these give a consistent view. *Pisum* is very diverse and its diversity is structured, showing a range of degrees of relatedness that reflect taxonomic identifiers, ecogeography, and breeding gene pools. However, as pointed out by Maxted and Ambrose (2001), the whole of *Pisum* is capable of genetic exchange, and the observations of Jing et al. (2005, 2010) and Vershinin et al. (2003) showed that allelic introgression between very diverse material does occur, so biologically there is good reason to consider *Pisum* as one species. This means that tree-like descriptions of the pattern of variation can be misleading because different markers have different trees. For this reason, a range of alternative approaches have been used, such as Principal Coordinate or Component analyses and modeling methods, such as Structure (Pritchard et al. 2000 and citations above). One such multivariate analysis, for a wide set of *Pisum* accessions listed in Table 12.1, is shown in Fig. 12.3c. This display is similar to the way that microarray data are presented, clustering by relationship along two independent axes. Accessions with similar genotype are in adjacent columns, while markers with similar allele distributions among accessions are in adjacent rows. This type of display is not popular, and it is easy to see why: it highlights the fact

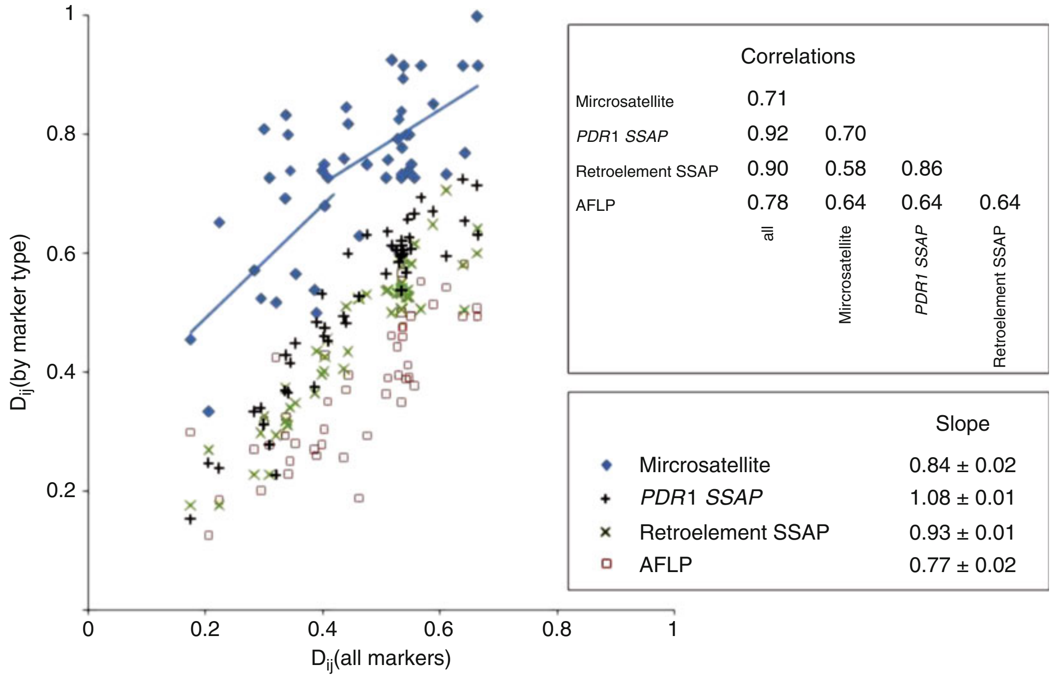


Fig. 12.2 Genetic distance and marker type. The data set illustrated in Figs. 12.2 and 12.3 comprises data from three marker types: Retrotransposon-derived SSAP (Pearce et al. 2000; Vershinin and Ellis 1999; Vershinin et al. 2003), AFLP (Ellis et al. 1998), and microsatellites. The graph plots all pairwise genetic distances between 50 accessions (see also Fig. 12.3). A total of 176 microsatellite bands were scored and the estimate of genetic distance based on these has been plotted against the genetic distance measure based on 1,104 pooled markers (◆). Similarly 176 of the PDR1 SSAPs (✚), 176 of the other retroelement SSAPs (✖) together with all the 101 AFLPs (□) were used for comparison. The correlation coefficient between all these measures is given as is the slope of the corresponding regression line. For the microsatellite comparison, two regression lines are plotted, the first with slope 0.95 ± 0.11 is for estimates below the mean on the *X*-axis and the second with slope 0.62 ± 0.05 is for estimates above the mean on the *X*-axis (*see text*)

that many markers give more or less the same information, and that many accessions are quite similar. In other words, the informative data sets are much smaller than the total amount of data.

P. abyssinicum accessions are very similar to each other, as is obvious from the (almost) solid red block on the top right of Fig. 12.3c. This clustering exaggerates the distinctness of *P. abyssinicum*; if there is only one *P. abyssinicum* accession then the multivariate analysis shows it as a member of the blue group, and *P. fulvum* becomes distinct. In three dimensions (explaining 45% of the variance), the points lie on the surface of a tetrahedron, with *P. abyssinicum* at one vertex and *P. fulvum* at another. The other accessions lie along the edge connecting the other two vertices, with a concentration of *P. sativum* at one end and *P. elatius* at the other. This is consistent with the idea that there are four main taxonomic groupings, one corresponds to *P. abyssinicum* and another to *P. fulvum*, while the other two represent admixtures between two poles, one corresponding roughly to *P. sativum* and the other to *P. elatius*. This pattern should be seen in Structure (Pritchard et al. 2000) analyses, in which four founder populations are proposed ($K = 4$). With the data set illustrated in Fig. 12.3, Structure repeatedly finds one of two alternative solutions (Fig. 12.4): one with these major taxa as the groups, while the other splits *P. sativum* geographically, combines *P. elatius* and *P. fulvum* and resolves *P. abyssinicum*.

The overall conclusion from these analyses of the pattern of diversity within *Pisum* is consistent with the taxonomic scheme of Maxted and Ambrose (2001) and the ILDIS database, except that these rank “*elatius*” as a subspecies of *P. sativum* but *P. elatius* and *P. sativum* would be better considered of equal rank. Vershinin et al. (2003) included minor taxonomic entities, such as *P. humile* and *P. jomardii*

Table 12.1 *Pisum* accessions and designations

Accession	Species designation	Continent of origin
JI156	*P. sativum*	Africa
JI281	*P. sativum*	Africa
JI189	*P. sativum*	
JI399	*P. sativum*	
JI188	*P. sativum*	
JI284	*P. sativum*	
JI52	*P. sativum*	
JI1030	*P. sativum*	
JI2546	*P. sativum* ssp. *transcaucasicum*	
JI2547	*P. sativum* ssp. *transcaucasicum*	
JI804	*P. sativum*	Asia
JI201	*P. sativum*	
JI209	*P. sativum*	
JI250	*P. jomardii*	
JI185	*P. sativum*	Africa
JI95	*P. sativum*	Asia
JI181	*P. sativum*	Asia
JI1033	*P. sativum*	Asia
JI2545	*P. sativum*	Asia
JI1846	*P. sativum*	
JI109	*P. sativum*	Asia
JI1854	*P. sativum*	Asia
JI2713	*P. sativum*	
JI1428	*P. sativum*	Asia
JI1346	*P. sativum*	Asia
JI45	*P. sativum* ssp. *transcaucasicum*	
JI241	*P. sativum*	Asia
JI102	*P. sativum*	Asia
JI196	*P. sativum* ssp. *transcaucasicum*	
JI85	*P. sativum*	Asia
JI2201	*P. sativum* ssp. *elatius*	
JI199	*P. sativum* ssp. *elatius*	
JI1794	*P. sativum* ssp. *humile*	
JI1074	*P. sativum* ssp. *elatius*	
JI261	*P. sativum* ssp. *elatius*	
JI262	*P. sativum* ssp. *elatius*	
JI1096	*P. sativum* ssp. *elatius*	
JI64	*P. sativum* ssp. *elatius*	
JI1093	*P. sativum* ssp. *elatius*	
JI254	*P. sativum* ssp. *elatius*	
JI1006	*P. fulvum*	
JI1010	*P. fulvum*	
JI2055	*P. sativum* ssp. *elatius*	
JI1092	*P. sativum* ssp. *elatius*	
JI1796	*P. fulvum*	
JI2385	*P. abyssinicum*	
JI130	*P. abyssinicum*	
JI1556	*P. abyssinicum*	
JI225	*P. abyssinicum*	
JI2	*P. abyssinicum*	

Accessions are on the order of PC1 of Fig. 12.4a and of Fig. 12.3c

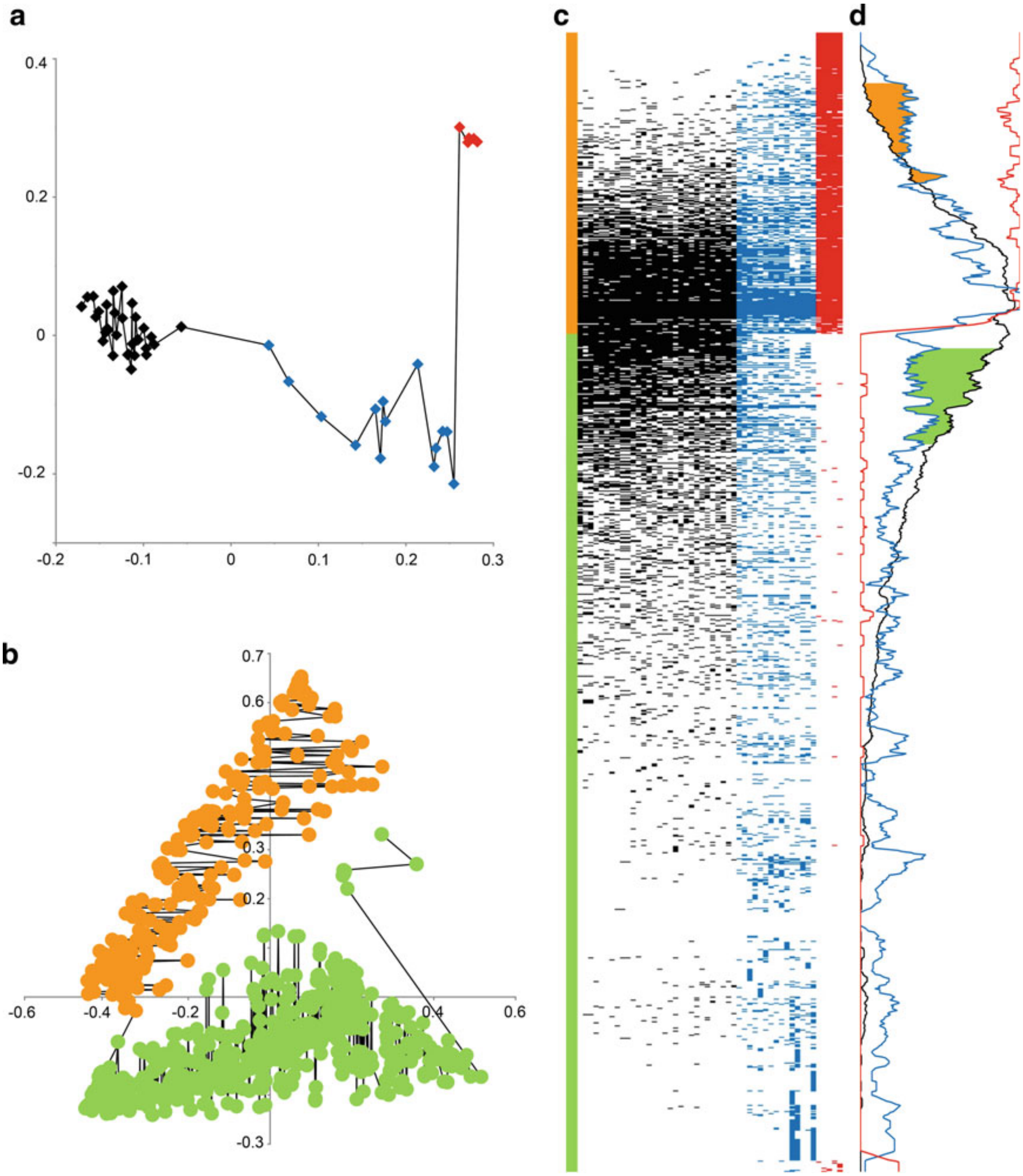


Fig. 12.3 Distribution of genetic diversity in *Pisum*. Pooled data for 1,104 markers from Ellis et al. (1998, 2005), Jing et al. (2005), Pearce et al. (2000), Vershinin and Ellis (1999) and Vershinin et al. (2003), together with data from 176 microsatellite markers (Fig. 12.2), scored on a set of 50 *Pisum* accessions was analyzed by multifactorial analysis using the Darwin 5 package (Perrier et al. 2003). (**a**) The relationship between accessions, given the marker data, is summarized for the first two dimensions that explain 23% and 12% of the variation, respectively. On average, one dimension is expected to explain ~2% of the total variation. Three main groups are identified by color, and a line threads between them in the order of PC1 (the *X*-axis). The accessions are listed in this order in Table 12.1. *Black points* correspond to *P. sativum, blue* to wild species, and *red* to *P. abyssinicum*. (**b**) The relationship between markers, given the accession data, is summarized for the first two dimensions that explain 9.6% and 6.3% of the variation, respectively. On average, one dimension is expected to explain ~0.1% of the total variation. The markers fall into two main classes and these are colored *orange* and *green*, respectively. A line threaded through these data follows descending values of PC2 (*Y*-axis) among the *orange*, and ascending values of PC1 (*X*-axis) for the *green*. (**c**) Marker data is shown. The columns correspond to accessions and rows to markers.

within *P. elatius*, as they were no more distinct genetically than individual *P. elatius* accessions. *P. elatius*, in either sense, includes a greater diversity than *P. sativum*. As *P. sativum* is the cultigen, domesticated from a wild ancestor, probably a type (or types) of *P. elatius*, it would seem more likely that *P. sativum* is subordinate to *P. elatius*. The etymology of *P. sativum* ssp. *elatius* is also a little odd as this is emphatically not a tall cultivated pea! Nevertheless, it is clear that *P. sativum* has distinct properties, is the most abundant type, and is usefully considered as a discrete entity, justifying its identification as a species. The distinctness of *P. sativum* and *P. abyssinicum* has led several authors to conclude that they were independently domesticated (Ellis et al. 1998; Vershinin et al. 2003; see also Baranger et al. 2004). Thus, in the full knowledge that genetic exchange within *Pisum* is possible, and has occurred, it seems reasonable to conclude that *Pisum* is a species complex with four subgroups; *P. fulvum* and *P. elatius* as wild taxa and *P. sativum* and *P. abyssinicum* as cultivated species.

12.4 Genetic Resources in Wild *Pisum*

There are 16 major germplasm collections for *Pisum* (Smýkal et al. 2008c), with some degree of overlap. These are dominated by cultivated forms, and although wild forms in these collections are highly diverse (Jing et al. 2010), they probably sample wild forms poorly. Furthermore, it is not known what proportion of existing alleles in wild material has been collected. A related issue is that the degree of inbreeding in wild material is unknown. The instances where useful alleles have been obtained from wild accessions thus probably under-represent the potential that wild material has for crop improvement. In the text that follows, some instances of useful allelic variation that have been found in diverse germplasm are described. Some landrace material of *P. sativum* and *P. abyssinicum* are also included as though they were "wild" because these are exotic to the bulk of cultivated *Pisum*.

12.4.1 Virus Resistance

The identification of virus resistance genes in pea is exemplified by the study of resistance to pea seed-borne mosaic virus and it is clear that the two recessive resistance genes *sbm1* and *sbm2* (Gao et al. 2004) encode eukaryotic initiation factor 4E proteins eIF4E and eIF(iso)4E, respectively (Gao et al. 2004a, b). The recessive resistance allele *sbm1* has been described in several accessions recorded as *P. sativum* from Ethiopia (Smýkal et al. 2010 also characterized the allele in many Indian accessions); several allelic forms exist, where amino acid substitutions correspond to distinct strain specificities.

12.4.2 Bacterial Interactions

Infection by *Pseudomonas syringae* pv. *pisi* is controlled genetically by race-specific resistance alleles, many of which are available in cultivars. The *Ppi2* allele conferring resistance to race 2 was also found in the exotic Ethiopian *P. sativum* JI281 (Hunter et al. 2001) and a race non-specific resistance has been detected in *P. abyssinicum* (Elvira-Recuenco 2000; Elvira-Recuenco and Taylor 2001). Interestingly, the symbiotic interaction between pea and rhizobia also has some strain specificity governed by the *sym1* or *sym2* alleles at the *Sym2* locus (Kozik et al. 1995). This

Fig. 12.3 (continued) A colored bar at the intersection of a row and a column indicates that the corresponding amplicon is present in that accession. Accessions are ordered left to right according to their position in PC1 in (**a**) (see Table 12.1), and the bars are colored according to the group colors in A. The markers are ordered top to bottom according to the threading line in (**b**), and a colored bar to the left indicates the marker group. (**d**) The frequency of band presence in each group is shown by a line colored corresponding to the group in A. To smooth this line, these are running averages of ten adjacent markers in (**c**). Two groups of markers are indicated by shaded areas: The orange area indicates amplicons rare (occupied site allele frequency less than 0.4) in *P. sativum* with respect to the wild *Pisum* species but common (occupied site allele frequency greater than 0.4) in *P. abyssinicum*. Those highlighted in *green* conversely are common in *P. sativum* with respect to the wild *Pisum* species but rare in *P. abyssinicum*. These markers are indicators of the distinct ancestry of these two domesticated types

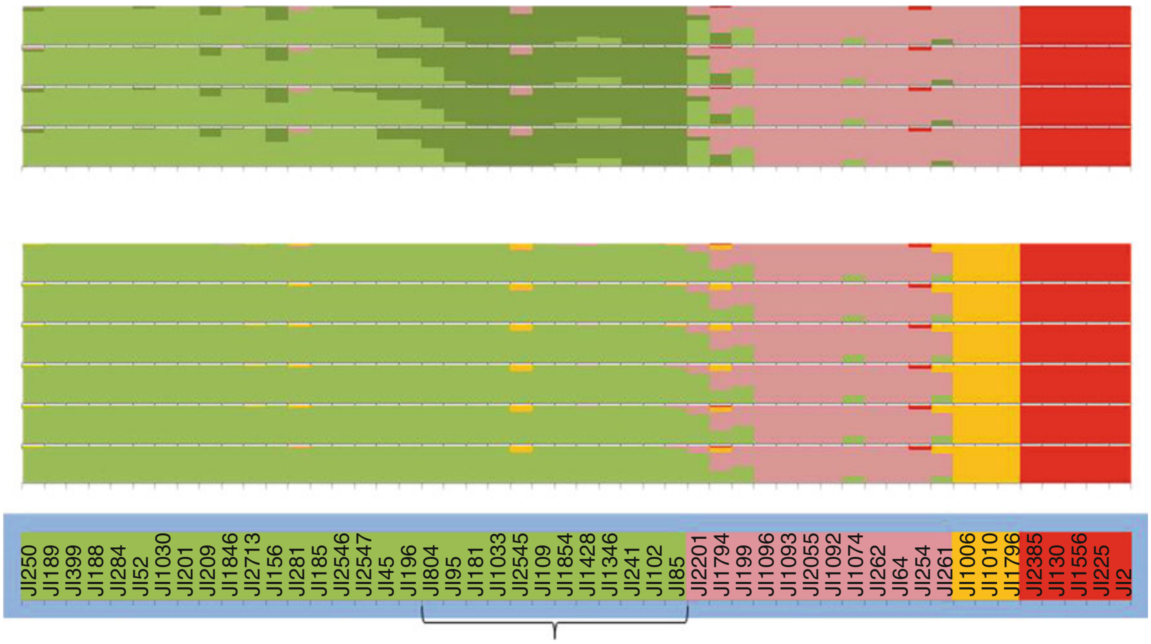


Fig. 12.4 Structure plots. The program Structure was used to analyze the data shown in Fig. 12.3, assuming four ancestral populations ($K = 4$). Ten repetitions of the analysis with 10,000 "burn-in" runs followed by 10,000 MCMC simulations were performed and the results presented as a *horizontal bar*. The proportion of the ancestry of each accession assigned to each progenitor population is indicated by *vertical colored bars*. For the four runs illustrated by the upper four panels, the assignments are to *red*, *pink*, *light green*, and *dark green* progenitors in a consistent pattern. For the six runs shown below, the patterns were again consistent but different. At the bottom, the accessions are identified and colored corresponding to taxonomic group: *red* – *P. abyssinicum*, *orange* – *P. fulvum*, *pink* – *P. elatius*, and *green* – *P. sativum*. The bracketed set of *P. sativum* accessions is from a restricted geographical area in Asia

specificity requirement for an acetylated nod factor appears to be determined by variant (or variants or possibly haplotypes) of a LysM domain receptor kinase (Limpens et al. 2003). Although the *sym1* allele is found in peas from Iran, the *sym2* allele (from Afghanistan) is associated with a distinct ecotype of *P. sativum* (Young and Matthews 1982) that appears to be genetically very distant from conventional *P. sativum* (Jing et al. 2010).

12.4.3 Resistance to Fungal Diseases

Major fungal diseases of pea include *Ascochyta pisi*, *Mycosphaerella pinodes* (*Ascochyta pinodes*), and *Phoma medicaginis*. These form a disease complex that affects the leaves and stem. No clear genetic resistance has been found. Zhang et al. (2006) surveyed a range of USDA accessions for resistance to isolates of *M. pinodes*. Most of the resistant types these authors identified had only partial resistance and appear to be *P. sativum*. In a survey of 78 *Pisum* accessions, Fondevilla et al. (2005) reported that the highest level of resistance was found in *P. fulvum* followed by *P. elatius* and then in *P. sativum* ssp. *syriacum*, and these authors reported that crosses with these material had been successful. Interestingly Le May et al. (2008) reported that, in field tests of winter peas, the allelic state of the *Hr* locus was associated with disease severity. *Hr* is an important regulator of the sensitivity of flowering time to photoperiod (Murfet 1973) and most cultivars carry the recessive insensitive allele *Hr*. In a study by Le May et al. (2008), it was the more sensitive (*Hr*) types that had greater resistance, so it will be important to determine whether improved resistance in wild *Pisum* can be separated from *Hr*.

Powdery mildew resistance in pea is caused by *Erysiphe pisi*, and good genetic resistance is available from recessive alleles at the *Er1* and *Er2* loci (Fondevilla et al. 2006). All of these resistance alleles are

already available within *P. sativum*, although at least one source is from exotic Chinese *P. sativum* germplasm. A range of useful alleles may be available in more extended exotic germplasm. Fondevilla et al. (2007) report the discovery of a new locus *Er3* in *P. fulvum*, where the dominant allele confers resistance. Given the sterility problems in *P. sativum* × *P. fulvum* crosses, and the difficulty in determining the allelism between a dominant and a recessive allele for a trait, it remains a possibility that *Er3* may yet turn out to be a novel allele of *Er1* or *Er2*.

12.4.4 Resistance to Oomycetes

A major problem that affects the inclusion of pea in crop rotations, especially in Europe, arises because of infection by *Aphanomyces eustiches*. Genetic resistance has been difficult to find, but quantitative trait loci have recently been described that confer some resistance (Pilet-Nayel et al. 2005); however, the crosses involved parents, all of which have been designated as *P. sativum*.

12.4.5 Pest and Parasite Resistance

Bruchid beetles cause considerable damage to grains in storage and may also infest developing seeds, causing quality and/or yield loss. This system has been studied in pea, with two interesting sources of potential resistance. The dominant *Np* allele (Snoad and Matthews 1969) has the property of causing neoplasms to develop on the pod surface, but UV light inhibits their formation. Thus, neoplasm formation tends to occur on shaded pods. The dominant allele is widespread in the genus, but tends not to be found in modern cultivars. Probesting and colleagues (Doss et al. 2000) showed that the *Np* allele conditions a reaction to a small molecule (bruchin) secreted by bruchid eggs, such that neoplasm outgrowth is initiated at the point of contact between the egg and the pod wall. It is further suggested that once the egg hatches, the larva eats the connecting outgrowth and is therefore detached from the pod, which is a potentially elegant mechanism of partial resistance. Byrne and colleagues (Byrne et al. 2000, 2008; Clement et al. 2002, 2009) have also shown that bruchid resistance, both pod based and seed based, can be found in some, but not all, *P. fulvum* accessions.

Orobanche cernata (broomrape) is a significant pest of legume crops in the Mediterranean area and is very difficult, if not impossible, to control using pesticide treatments, so the availability of genetic resistance would be a significant boon. Surveys of germplasm (reviewed by Rubiales et al. 2009) have uncovered partial resistance in *P. abyssinicum*, *P. elatius*, and *P. fulvum* as well as some *P. sativum* accessions, but singly, none of these provides significant resistance. This problem may be very deep as recent studies have connected mycorrhization (a symbiosis generally considered to have been important for plant colonization of the land), bud outgrowth (an essential component of plant growth), and strigolactone, the germination stimulant for *Orobanche* (Gomez-Roldan et al. 2008; Umehara et al. 2008). One possibility is that natural variation in strigolactone secretion by roots may be an important determinant of susceptibility to broomrape infection, suggesting an analysis that could be undertaken of existing sources for partial resistance.

12.5 General Conclusions

We have seen that *Pisum* is a diverse genus and that its subdivision into wild vs. cultivated species is unclear. Nevertheless, a substantial amount of genetic variation remains available in non-cultivated *Pisum*. Plant breeders are understandably reluctant to approach wild germplasm because there are many more ways to impair performance genetically than to improve it. Although there may be some useful alleles in wild material, we can expect that there are also many detrimental alleles. When time is short, and funds are limited, it is simplest to proceed by surveying germplasm with as few detrimental alleles as possible. The diversity of *P. sativum* has meant that this is generally a reasonably successful strategy, but as we have seen, there are cases where alleles are required that are not currently available in breeders' lines.

There are two general approaches to providing novel and useful alleles to breeders' lines: transgenic methods are one, but these are expensive to devise, while the other is the survey of non-adapted material for useful alleles. These strategies are not mutually

exclusive. The introgression of a useful allele from a wild relative may be most easily achieved by transgenesis as was the case in potato for the *RB* gene from *Solanum bulbocastanum* that conferred resistance to *Phytophthora infestans* (Song et al. 2003).

It seems unlikely that transgenic methods for pea improvement will be either practicable or acceptable to consumers for some time. So alleles to be deployed in pea improvement must come from cross-compatible *Pisum*, yet we want to avoid the introgression of detrimental alleles. For this reason, it would seem appropriate to initiate programs for the generation of chromosomal segment substitution lines based on a selected set of diverse *Pisum* using a common, adapted background for the recurrent parent.

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Chapter 13
Trifolium

W.M. Williams and S.N. Nichols

13.1 Introduction to *Trifolium*

The clover genus *Trifolium* has 250–300 species (Allen and Allen 1981; Zohary and Heller 1984; Ellison et al. 2006), about 10% (20–30) of which are used as forage plants in commercial agriculture, and a greater number are used locally for fodder in their native zones. This chapter will give most consideration to the wild relatives of the most important of these species, white clover (*T. repens* L.), red clover (*T. pratense* L.), and berseem (*T. alexandrinum* L.). However, there are nearly 50 other species, which are listed here as being of current or potential interest, either for direct use in agriculture, or as sources of genes for other species. Clovers, like most legumes, are co-evolved complexes of plant, symbiotic bacteria, fungi, and insect pollinators. Strains of symbiotic N-fixing bacteria (*Rhizobium leguminosarum* biovar *trifolii*) have co-evolved with plant species and populations, and form an important part of the species adaptations. These are mentioned, but have not been comprehensively reviewed. Similarly, pollination and mycorrhizal associations are indicated only where they are of special significance. We have based our classification of species relatedness on the DNA phylogenetic analysis of Ellison et al. (2006), which replaces the former morphological classification of Zohary and Heller (1984).

13.2 Genetic Resources of *Trifolium*

Germplasm exploration and conservation of *Trifolium* species was reviewed by Gillett and Smith (1985). Conservation initiatives in recent years have been led by N.L. Taylor (University of Kentucky), S. Greene (USDA), R.R. Smith (University of Wisconsin), R. Snowball (Agriculture Western Australia) and W.M. Williams (AgResearch, New Zealand).

Many *Trifolium* species have very narrow distributions and must be considered to be under threat in their native environments. All of them have orthodox seed that can be dried and stored for long periods at low temperature, especially when hard. However, many are not well represented in seed bank collections. This applies especially to many of the North American species that tend to be distributed in small isolated pockets that are hard to access. They need further urgent collection, along with those of the Mediterranean region, many of which are threatened by overgrazing and increasing environmental change.

Even the wild relatives of major species are poorly represented in seed banks and need more extensive collecting. For example, the numbers of accessions of white clover relatives in major seed banks are given in Table 13.1. The most threatened species is *T. occidentale* Coombe, which occurs in relatively precarious coastal habitats, and may be vulnerable to drought, soil erosion, tidal incursion and property development. It is important that this species is well collected and stored in seed banks. Some species are little represented in seed banks, e.g., *T. pallescens* Schreb.

13.3 The Wild Relatives of White Clover (*T. repens*)

White clover (*T. repens*) is a tetraploid ($2n = 4x = 32$) stoloniferous herb that is native to, and widespread in, Europe, Eurasia, the Middle East and North Africa. It has been introduced as a cultivated species to many

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Table 13.1 Numbers of accessions of the wild relatives of white clover in major seed banks

Species	Accessions in seed banks[a]	Comments
T. ambiguum	GRIN > 100 WPBS 2	
T. occidentale	GRIN 2 KEW 1 MFFGC (NZ) 50	A threatened species in several locations
T. nigrescens	GRIN 42 WPBS 10 KEW 6 MFFGC (NZ) 23	
T. uniflorum	GRIN 3 MFFGC (NZ) 2	A very diverse species that lacks collections
T. pallescens	GRIN 3 MFFGC (NZ) 3 WPBS 3	Little collected and requires attention
T. thalii	GRIN 4 WPBS 2	

Similar situations apply to the other wild relatives.
[a]GRIN = USDA system; WPBS = Welsh Plant Breeding Station, Aberystwyth; KEW = Millennium Seed Bank, London; MFFGC (NZ) = Margot Forde Forage Germplasm Centre, New Zealand

countries of the world for use as a pasture plant (Zohary and Heller 1984; Williams 1987; Tesfaye and Williams 2008). It thrives best on damp soils and in grassy places, and is a very diverse species, with adaptations to a range of environmental niches. Zohary and Heller (1984) subdivided it into eight botanical varieties, based on degree of hairiness, organ (especially leaf) size, peduncle length and calyx venation.

White clover is a tetraploid ($2n = 4x = 32$) species with disomic (amphidiploid) inheritance (Williams et al. 1998), and is likely to be of hybrid origin (Ellison et al. 2006). Its 1C-genome size has been reported as 0.559 pg (Vizintin et al. 2006). Despite its considerable genetic diversity (Caradus et al. 1989), white clover lacks variation for traits that would improve its adaptation to a wider range of environments, especially semi-arid soils of low fertility (Williams et al. 2007). It is already the most used temperate legume of grazed pastures, but would be used more widely if its gene pool could be enhanced using wild relatives.

On the basis of DNA sequence phylogeny, Ellison et al. (2006) placed white clover in a new section *Trifoliastrum* S.F.Gray, along with its closest relatives (Table 13.2). These species are very diverse, ranging from annuals to long-lived perennials, and offer potential genetic resources for genome elucidation and improvement.

Table 13.2 The species closely related to white clover (section *Trifoliastrum*)

Species	Ploidy[a]	Annual/perennial
T. nigrescens	$2x$	Annual
T. occidentale	$2x$	Perennial
T. uniflorum	$4x$	Perennial
T. isthmocarpum	$2x$	Annual
T. thalii	$2x$	Perennial
T. pallescens	$2x$	Perennial
T. retusum	$2x$	Annual
T. suffocatum	$2x$	Annual
T. parnassi	$2x$	Perennial
T. cernuum	$2x$	Annual
T. glomeratum	$2x$	Annual
T. ambiguum	$2x$, $4x$, $6x$	Perennial
T. montanum	$2x$, $4x$	Perennial

[a]Cleveland (1985)

13.3.1 T. nigrescens *Viv.*

13.3.1.1 Basic Botany

T. nigrescens is a diploid annual species that occurs as at least three subspecies (Williams et al. 2001) from western Europe to Asia Minor. *T. nigrescens* ssp. *nigrescens* is distributed across the western Mediterranean countries of Europe, Turkey, Crete and North Africa in fields, and among shrubs on stony hillsides and in damp places (Zohary and Heller 1984). It has sprawling stems

up to 1 m long from a single crown, white or pink flowers, and differs from the other subspecies because of its elongated pod with 3–4 or more seeds. *T. nigrescens* ssp. *petrisavii* (Clem.) Holmboe occurs in the eastern Mediterranean, from Greece to Turkey, and in the Middle East and the Caucasus. It resembles ssp. *nigrescens,* but has a 1–2-seeded pod. It occurs from sea level to 1,100 m on sand dunes, river banks, roadsides, fields, dry hillsides, and moist places. A giant form, with thick, hollow stems, large leaflets, and 1–2 seeded pods was recognized by Hossain (1961) as *T. nigrescens* ssp. *petrisavii* var. *meneghinianum* (Clem.) Hossain. It is found in damp places in Northwest and South Turkey, Lebanon, and the Caucasus. This form was not recognized by Coombe (1968) or Zohary and Heller (1984), but it probably deserves subspecies status as it differs significantly from ssp. *petrisavii* in crossability with other taxa, and in its distribution of rDNA on the chromosomes (Williams et al. 2001). A form designated *T. nigrescens* ssp. *petrisavii* var. *grandifolium*, described from Southwest Turkey by Ertekin and Akbayin (2000) is probably the same taxon.

All three subspecies have $2n = 2x = 16$ chromosomes. As with the other species closely related to white clover, the chromosomes are small and predominantly metacentric or submetacentric and not reliably distinguishable except for one satellited (NOR-bearing) pair (Chen and Gibson 1971a; Ansari et al. 1999). Chen and Gibson (1971a) found that the karyotype of ssp. *meneghinianum* differed from the other two subspecies, particularly in having larger satellited chromosomes. This suggested that ssp. *nigrescens* and *petrisavii* are more closely related to each other than to ssp. *meneghinianum*. The genome size of *T. nigrescens* is small (1C haploid nuclear genome value = 0.39 pg; Vizintin et al. 2006). In the same study, the 1C value for *T. repens* was 0.559.

Among the three subspecies, to-date only *T. nigrescens* ssp. *petrisavii* has been commercialized. It is used as a winter annual forage crop in the southern States of the United States of America, where it is known as “ball clover” (Knight 1985). It produces up to 7.5 MT/ha of high protein (18–26%) forage. It has very high seed production (200–600 kg/ha), of which about half is hard seed that stays in the soil and germinates in subsequent years. The other subspecies occur naturally throughout their distributions, and are almost certainly part of meadows that are grazed and browsed by domestic animals. A strain of symbiotic nodulating bacteria, *R. leguminosarum* biovar *trifolii* belonging to the *T. subterraneum* L. group was more effective on ssp. *petrisavii* than other strains (Pryor and Lowther 2002), including one from *T. repens*.

13.3.1.2 Role in Classical and Molecular Genetics Studies

Classical genetic analyses have shown that the self-incompatibility of *T. nigrescens* is controlled by a gametophytic system of oppositional *S* alleles (Brewbaker 1955). Chromosome doubling of *T. nigrescens* led to some self-fertile plants in the F_2 generation following open-pollination of the self-incompatible doubled plants. This was attributed to competition interaction between alleles in heterogenic pollen (Brewbaker 1955).

T. nigrescens ssp. *nigrescens* is predominantly cyanogenic. However, the existence of some acyanogenic genotypes in a few populations has allowed the genetics of cyanogenesis to be studied. Williams and Williamson (2001) showed that *T. nigrescens* has a two-locus genetic system, with one locus (*Ac*) controlling cyanogenic glucoside development, and the other (*Li*) controlling production of the hydrolyzing enzyme. This is the same system as in white clover, and led these authors to conclude that *T. nigrescens* was a likely donor of a genome to white clover. Other evidence that the *Li* locus in *T. repens* could have been donated by *T. nigrescens* was provided by Kakes and Hakvoort (1994), who compared the enzyme activities and found them similar in the two species, although immunology indicated slightly different structures.

All 16 *T. nigrescens* ssp. *meneghinianum* chromosomes have a multiple-copy centromeric satellite DNA repeat, TrR350, a feature in common with white clover and some other closely related species. By contrast, *T. nigrescens* ssp. *nigrescens* shows the same repeat in much lower copy-number on all chromosomes (Ansari et al. 2004).

13.3.1.3 Role in Crop Improvement

Traditional breeding: There are no available cultivars of *T. nigrescens* in the USA, a local form is marketed as “ball clover” mainly by one company.

Interspecific hybrids: *T. nigrescens* has been used by several workers to achieve interspecific hybrids with white clover.

T. repens (8*x*) × *T. nigrescens* (4*x*): Among the first successful interspecific crosses in *Trifolium*, were reciprocal crosses of colchicine-doubled *T. repens* and *T. nigrescens* to give two fertile hexaploid hybrids (Brewbaker and Keim 1953). One was self-fertile, and the other self-incompatible. Both hybrids were interfertile with one another and both parents and produced progeny with a range of ploidy levels (5*x*–7*x*). One was very short-lived and the other, although prostrate and rapidly spreading, did not have nodal roots.

T. repens (4*x*) × *T. nigrescens* (2*x*): The cross between *T. repens* and *T. nigrescens* ssp. *nigrescens* can be easily made without the use of embryo rescue to give triploid F_1 hybrids. Early reports of hybrids were from Keim (1953), Hovin (1962) and Chen and Gibson (1970a). Pairing of *T. repens* and *T. nigrescens* chromosomes was reported by Chen and Gibson (1970a). Although seed set was very high when *T. nigrescens* ssp. *nigrescens* was used as the seed parent (Williams et al. 2001), progeny plants arising from *T. nigrescens*-derived seeds were often weak. The cross was, therefore, more successful when *T. repens* was used as the seed parent. Crosses of *T. nigrescens* ssp. *petrisavii* and ssp. *meneghinianum* as female with *T. repens* as male gave much lower numbers of seeds (Williams et al. 2001).

Crosses with *T. nigrescens* ssp. *nigrescens* have been used to transfer clover cyst nematode resistance (Hussain et al. 1997a), root-knot nematode resistance (Pederson and Windham 1989), and high inflorescence production and seed-set (Marshall et al. 1995) from *T. nigrescens* to white clover. A ploidy series (2*x*–7*x*) involving different balances of hybrid genomes was developed by colchicine doubling a sterile 3*x* hybrid plant to produce a fertile 6*x* plant and backcrossing this to the parents (Hussain et al. 1997b). The 3*x* and 6*x* hybrids were as resistant to clover cyst nematode as the most resistant *T. nigrescens* genotypes (Hussain et al. 1997a). To increase flowering and seed-set, Marshall et al. (1995) generated triploid F_1 hybrids and backcrossed these repeatedly to *T. repens* to get *T. repens*-like backcross hybrids introgressed by *T. nigrescens* (Marshall et al. 1998, 2002a, b, 2005). Amplified fragment length polymorphism (AFLP) markers were employed to monitor the introgression of the seed yield trait (Marshall et al. 2003b). The hybrids had N-fixation levels similar to white clover (Abberton et al. 1999), and similar digestibility with higher soluble carbohydrate and slightly lower protein concentrations than white clover (Marshall et al. 2003c). Higher seed yield was transferred from *T. nigrescens* most successfully to medium and large leaved white clover varieties, while the small-leaved variety was not significantly improved (Marshall et al. 1999, 2008).

13.3.2 T. occidentale *(Western Clover)*

T. occidentale is a diploid ($2n = 2x = 16$) perennial species that occurs only on the gulf stream coasts of western Europe and outlying islands, from north-western Portugal and Spain, to south-eastern Ireland. It occurs on the western coast of France, near Land's End in Cornwall and in western Wales. It is adapted to a relatively narrow range of maritime habitats, from beach sand above high tide and consolidated sand dunes to cliff tops immediately adjacent to the ocean. It is predominantly self-pollinating, but populations from north-western Spain are cross-pollinating. The self-pollinating populations from France, United Kingdom and Ireland are relatively uniform (Coombe 1961) and appear to have little genetic diversity. By contrast, the cross-pollinating populations from Spain have high genetic diversity among and within populations (Williams et al. 2009).

It is a stoloniferous species, resembling a small white clover. Indeed, this resemblance is so close that the species was not identified as a unique taxon until about 1960 (Coombe 1961). There is a set of characteristics which, in combination, can be used to distinguish *T. occidentale* from *T. repens*, (Coombe 1961). Briefly, relative to white clover, *T. occidentale* has short stolons, small, thick leaflets, often wider than long, with a very glossy under-surface. Leaf veins are few, and are not translucent when held up to the light. Petioles, petiolules and peduncles are hairy. Heads have few florets (less than 20–40) and creamy-white petals (never pink).

T. occidentale is taxonomically distinct from *T. repens* (Coombe 1961). It is diploid, has a distinctive habitat, and hybrids with white clover are unknown in nature. There is a possible uncertainty of distinctness from *T. biasolettii* Steud. and Hochst., a similar Mediterranean taxon with hairy petioles but pink flowers. Coombe (1961) presented evidence that

T. occidentale was distinct from *T. biasolettii* which he concluded to be close to *T. repens*. However, Zohary and Heller (1984) did not distinguish the two, and classified both *T. biasolettii* and *T. occidentale* as *T. repens* var *biasolettii* (Steud. and Hochst.) Asch. and Graeb. More information is needed on *T. biasolettii*, especially its ploidal level, before its status can be resolved. However, there is no doubt that *T. occidentale* is a separate species from *T. repens*.

The karyotype of *T. occidentale* is similar to that of *T. nigrescens* (Chen and Gibson 1971a; Ansari et al. 1999), consisting of two metacentric, five submetacentric and one satellited subtelocentric chromosome pair. The genome size is half that of white clover (Williams et al. 2009).

T. occidentale has never been domesticated and, in its native state, it contributes very little to agriculture. The author has collected it in its natural habitat in grazed pastures near ocean-facing cliff-tops on the Gower Peninsular, Wales, but found that it was replaced about 50 m inland by white clover. Elsewhere, its natural habitat is too close to the sea for agricultural use. Because of its diploid, self-pollinating nature, it has been developed as a model species for genetic and genomic research relating to the white clover complex (Williams et al. 2009). It is also a likely ancestral diploid parent of white clover (Ellison et al. 2006; Hand et al. 2008; Williams et al. 2009).

T. repens is polymorphic for cyanogenesis, a trait controlled by two disomic genetic loci giving four different phenotypes (Corkill 1942). One of the loci (*Ac*) controls presence or absence of the cyanogenic glucosides linamarin and lotaustralin, while the second locus (*Li*) controls presence or absence of the ß-glucosidase enzyme, linamarase. *T. occidentale* is polymorphic for cyanogenic glucosides but no plant has ever been found with linamarase (Kakes and Chardonnens 2000). This has several implications for the evolution of white clover and for the systematic separation of *T. repens* and *T. occidentale*, which occur naturally adjacent to each other. The absence of linamarase from *T. occidentale* strongly suggests that the *Li* allele in *T. repens* was not donated by *T. occidentale*, despite this species being one of the parental ancestors. As the other putative parent, *T. pallescens*, is also acyanogenic (from limited testing so-far), the source of the linamarase allele is a mystery. One possibility is that it came from *T. nigrescens*, the only other strongly cyanogenic *Trifolium* species, as discussed above. Because *T. nigrescens* (2*x*) and *T. occidentale* (2*x*) are weakly interfertile and may form hybrids (Williams et al. 2008), a first generation hybrid between these diploids may have been involved in the origin of white clover. However, there is no evidence for this, and because the first generation hybrids can backcross to *T. nigrescens* but not *T. occidentale*, the transfer of genes from *T. nigrescens* to *T. occidentale* is unlikely by this route.

13.3.3 T. ambiguum *M. Bieb. (Caucasian Clover, Kura Clover)*

13.3.3.1 Basic Biology

T. ambiguum is a very long-lived perennial species found in Romania, Turkey, Armenia, the Caucasus, Iran and Iraq. It occurs at relatively high altitudes from 1,700 to 2,750 m on steep slopes, stream edges, etc. (Zohary and Heller 1984). Below ground it has rhizomes, and very thick, deep roots. Above-ground it has robust stems arising from rhizomes that do not root at the nodes. The flowers are white, turning pink after fertilization, so that the heads are white above and pink below. The flowers have a very pleasant scent and are excellent sources of nectar.

T. ambiguum occurs as a polyploid series, with diploid, tetraploid, and hexaploid populations. The different chromosome races are clinal in distribution (Kannenberg and Elliott 1962), with the hexaploids at the lowest altitudes and diploids at the highest. The tetraploids tend to be at intermediate altitudes.

Karyotype analysis of diploid *T. ambiguum* by Chen and Gibson (1971a) identified one pair of metacentric, six pairs of submetacentric and one satellited pair of chromosomes. The 1C nuclear genome size in hexaploid *T. ambiguum* was estimated by Vizintin et al. (2006) to be 0.764 pg, i.e. approximately double that of *T. nigrescens* and 36% larger than *T. repens*. *T. ambiguum* was placed with *T. repens* in section *Trifoliastrum* by Ellison et al. (2006) on the basis of DNA sequence phylogenetics. Formerly (Zohary and Heller 1984), it was classified in section *Lotoidea* Crantz., but in a different subsection from *T. repens*. (Subsection *Platystylium* Willk., on the basis of its having only basal florets deflexed after anthesis).

T. ambiguum is recognized as a potentially useful agronomic species in several countries, including Australia (Dear and Zorin 1985), New Zealand (Black and Lucas 2000), and USA (Brummer and Moore 2000), in addition to its region of natural distribution. To date, cultivars have been developed at diploid, tetraploid and hexaploid levels, but only the hexaploids have been commercialized. It is valued for its very persistent nature, drought tolerance, high forage quality and summer productivity (Hill and Mulcahy 1995). However, difficulties in obtaining adequate seed yields have led to high seed prices and these have hampered its commercialization. In addition, it is very slow to establish, allocating more carbon to root than shoot growth, and also having slow leaf and shoot development (Black et al. 2006). Consequently, establishment failures are frequent and create another source of difficulty in the uptake of this species.

13.3.3.2 Role in Crop Improvement

T. ambiguum has several desirable traits that breeders of white clover would like to introduce to the gene pool. These include rhizomes, deep roots, drought tolerance, and virus resistance (Townsend 1985). Crosses have proved to be very difficult to achieve.

T. ambiguum (4*x*) × *T. repens* (4*x*): Fertile hybrids have been obtained to date only by Williams and Verry (1981), who used embryo rescue, and Meredith et al. (1995) who used ovule culture. In both cases only one or two fertile F_1 plants resulted from large numbers of attempts. Yamada et al. (1989) used ovule culture and produced some hybrids, but these were of very low fertility and did not grow well in the field. The F_1 4*x*-H-435 produced by Williams and Verry (1981) was chromosome-doubled by Anderson et al. (1991) to produce 8*x*-H435. Backcrosses of 8*x*-H435 to white clover were produced by these workers and by Hussain and Williams (1997). The BC_1F_1 families were hexaploid (6*x*) with genomic constitution of AARRRR (A = *ambiguum*, R = *repens*), and have been included in a large breeding program to develop varieties of this genomic constitution (Widdup et al. 2003; Williams and Hussain 2008). Williams et al. (2006c) reported that the backcrosses of 8*x* hybrid material to *T. repens* was more successful when *T. repens* was used as the female parent. Apparent negative nucleo-cytoplasmic interactions occurred when the hybrid material was used as female, leading to poor vigor and frequent chlorophyll deficiencies in the progeny. The BC_1 hybrids have been advanced to at least BC_1F_6, and combine the stoloniferous growth habit of *T. repens* with the deep rooted character of *T. ambiguum*. To date, the general vigor and agronomic performance of these hybrids has limited their commercial potential.

The F_1 created by Meredith et al. (1995) was male sterile, but formed seeds when pollinated with *T. repens*. The resulting BC_1 plants were 6*x*, presumably as the result of the functioning of unreduced gametes from the F_1 hybrid. BC_2 and BC_3 progenies were produced and extensively characterized. The BC_3 (presumably aneuploid) hybrids showed a small amount of rhizome expression in plants that were otherwise similar in yield, persistence and N-fixation to white clover (Abberton et al. 1998, 2000; Marshall et al. 2003a). The BC_2 (presumably 5*x*) plants showed enhanced drought tolerance over white clover (Marshall et al. 2001). The forage quality of the backcrosses was excellent, and compared with white clover had similar digestibility, lower protein and higher soluble carbohydrate (Abberton et al. 2002; Marshall et al. 2004).

Using bulked segregant analysis, Abberton et al. (2003) were able to find AFLP markers that were associated with the *T. ambiguum* rhizomatous trait through backcrossing to white clover. This should enable successful marker-assisted selection for this trait, which is otherwise difficult to select as it is underground and is sometimes not expressed until after more than 1 year.

Partially fertile backcrosses to *T. ambiguum* were reported by Williams et al. (2006b). These workers obtained backcrosses at very low frequencies using embryo rescue. Success was achieved at two ploidal levels: *T. ambiguum* (2*x*) × 6*x* (*T. ambiguum* x *T. repens*) to give 4*x* AARR progeny, and *T. ambiguum* (6*x*) × 6*x* (*T. ambiguum* × *T. repens*) to give 6*x* progeny with genomic constitution AAAARR.

13.3.4 *T. uniflorum L.*

13.3.4.1 Basic Botany

T. uniflorum ($2n = 32$) is a perennial, wild species from the Mediterranean region, found in Greece, Turkey, southern France, southern Italy and Libya

(Zohary and Heller 1984). It is tolerant of dry environments, and occurs in coastal to inland habitats, including halophilous coastal communities (Brullo et al. 2000). The species is of no economic importance.

It is self-incompatible (Pandey 1957), although Gibson and Chen (1971), found some self-compatibility in three seed lines. Pandey (1957) determined the chromosome number of *T. uniflorum* to be 32. This was confirmed by Gibson and Chen (1971) who also found predominantly bivalent and quadrivalent chromosome pairing. It has four satellited chromosomes (Gibson and Chen 1971; Gibson et al. 1971) which, along with the chromosome pairing arrangements, was interpreted as evidence of an autotetraploid origin for *T. uniflorum* (Gibson and Chen 1971).

T. uniflorum appears to be highly variable. Vierhapper (1919) divided the species into seven varieties, based mainly on floral morphology. Hossain (1961) also described the species as variable, including leaflet size and shape, peduncle length, and pedicel length and breadth, in addition to floral characteristics. Greuter (1972) listed the co-existence of *T. uniflorum* with a mountain ecotype on the Greek island of Crete (*T. uniflorum* var. *breviflorum* Boiss.). Badr et al. (2002) evaluated genetic diversity in a range of *Trifolium* species and accessions, and found that *T. uniflorum* had the highest diversity and differentiation among accessions.

Some authors treat *T. savianum* Guss. as a synonym or subspecies of *T. uniflorum*, while others consider it to be a distinct species. Brullo et al. (2000) proposed that *T. savianum* is an endemic Sicilian species separated from *T. uniflorum* by geographic isolation, with the two exhibiting differing morphological characteristics and ecological associations. They speculated that *T. savianum* could have adopted a mountainous habitat prior to the ice-age, isolating it from low land connections in glaciations, which may have allowed genetic exchange in the eastern populations of *T. uniflorum*.

Detailed morphological descriptions of *T. uniflorum* are given by Zohary and Heller (1984) and Brullo et al. (2000). Its name derives from its production of groups of 1–3 large florets, in contrast to the inflorescences of other *Trifolium* species. Various authors have particularly noted its short internodes, thick and deep roots with a woody tap root, and a relatively large seed (Chen and Gibson 1971b; Pandey and Petterson 1978; Pandey et al. 1987). These characteristics were suggested to have potential to improve *T. repens* through interspecific hybridization. Gibson et al. (1971) suggested that the larger seed size could improve the seedling vigor of *T. repens*. The stronger deeper roots were also suggested to improve drought tolerance, pest tolerance, nutrient interception and soil conservation (Pandey and Petterson 1978; Pandey et al. 1987). Vierhapper (1919) noted that low nutrients or exposure to drought caused a decrease in the size of above ground plant parts (e.g., leaves, flowers and petiole length).

13.3.4.2 Conservation Needs

As indicated in Table 13.1, there are extremely limited numbers of seed accessions of *T. uniflorum* available in seed banks. The GRIN system reports only three available accessions. The Margot Forde Forage Germplasm Center reports only two accessions. Considering the great diversity of this species, the current ex situ conservation effort is very poor; it is essential that an effort is made to secure far more accessions in the seed banks of the world.

13.3.4.3 Nodulation

In laboratory studies, *T. uniflorum* has been found to form partially effective or effective nodules with three *R. leguminosarum* bv. *trifolii* strains from *T. subterraneum* L. and one from *T. medium* L. (Yates et al. 2003; Howieson et al. 2005). Those from *T. subterraneum* are commercial Australian *Trifolium* strains. Rhizobia from *T. repens* were not tested on *T. uniflorum*, but the two strains tested from *T. uniflorum* were ineffective on *T. repens*.

One strain of rhizobia from *T. uniflorum* was effective or partially effective with ten *Trifolium* species (Howieson et al. 2005). Of two other strains, one was effective and one ineffective with *T. fragiferum* L. (perennial) (Yates et al. 2003; Howieson et al. 2005). All successful relationships involving *T. uniflorum* were with European clovers and rhizobial strains isolated from European species. They included both perennial and annual clovers. *T. uniflorum* rhizobia were unsuccessful with the African, North American and South American species tested and, similarly, rhizobia isolated from clovers from these regions were unsuccessful with *T. uniflorum*.

13.3.4.4 Role in the Elucidation of the Origin and Evolution of White Clover

Badr et al. (2002) concluded that *T. uniflorum* is one of the ancestors of white clover, and Evans (1962a) also suggested this was possible given the success in hybridizing the two species. However, the results of a molecular marker based phylogeny of the *Trifolium* genus did not confirm this (Ellison et al. 2006). Nevertheless, *T. uniflorum* is within the new section *Trifoliastrum* designated by Ellison et al. (2006) and so is very closely related to *T. repens*. Hybridization of white clover with *T. uniflorum* could, therefore, be expected to be relatively successful compared to other more distantly related species, such as *T. ambiguum*.

Studies by Chen and Gibson (1970a, b) indicated homology between the chromosomes of *T. repens*, *T. occidentale*, and *T. nigrescens*, suggesting closely related genomes. Chen and Gibson (1972b) subsequently showed that *T. uniflorum* may share a similar genome to these three species. Ansari et al. (1999) found that these four species share a similar chromosome, bearing both a nucleolus organizer region and a large 5S ribosomal DNA repeat on opposite arms. *T. uniflorum* also shares a centromeric satellite DNA repeat TrR350 with these species (Ansari et al. 2004).

13.3.4.5 Role in Classical and Molecular Genetic Studies: Cyanogenesis

It is unclear whether *T. uniflorum* is cyanogenic. Gibson et al. (1971) found that *T. occidentale* contained cyanoglucoside but *T. uniflorum* did not. However, Gibson et al. (1972) later found two out of ten *T. uniflorum* plants tested did contain cyanoglucoside, although only trace amounts were detected. None of the *T. uniflorum* plants tested contained the hydrolyzing enzyme. The authors questioned the significance of the weak response of *T. uniflorum* and recommended that further tests be carried out using more accessions and greater sample sizes. Given the variability in morphological features observed in *T. uniflorum* by other authors it is possible that variability may also exist for cyanogenesis. Within *T. repens* there are certainly cyanogenic and acyanogenic genotypes, plus variation in the level of HCN produced (Crush and Caradus 1995). The variation in cyanogenesis with latitude found by Daday (1954) could also suggest that high levels may be expected, at least in some populations, in a Mediterranean species such as *T. uniflorum*.

13.3.4.6 Role in White Clover Improvement

Pest and Disease Tolerance

Dymock and Hunt (1989) found that grass grub (*Costelytra zealandica*) fed equally on *T. repens* and *T. uniflorum* in terms of percentage of root dry weight consumed. For both species, roots over 2 mm in diameter were eaten less than those under 2 mm, but this was particularly so for *T. uniflorum* (4.0% of root dry weight versus 38.1% for *T. repens*). Dymock et al. (1989) subsequently found that grass grub larval growth was reduced on *T. uniflorum* compared to other *Trifolium* species. Growth of several seedlines was comparable to, or lower than, *Lotus pedunculatus* Cav., a species known to be resistant to grass grub. The authors speculated that resistance of *T. uniflorum* could be due to nutritional quality of the roots, absence of feeding stimulants, or the production of feeding deterrents. Sutherland (1979) also suggested that the woody nature of *T. uniflorum* roots could provide a mechanical method of tolerance. Dymock and Hunt (1989) suggested the resistance of *T. uniflorum* to grass grub could be utilized by hybridization.

Gibson et al. (1971) also suggested that *T. uniflorum* may have some virus tolerance, listing only one virus as causing symptoms. These authors also reported a lower effect of sooty blotch and powdery mildew on *T. uniflorum* compared to *T. occidentale*.

Pederson and Windham (1989) studied the resistance of eight *Trifolium* species to southern root-knot nematode (*Meloidogyne incognita*). Four species had lower mean gall indexes than *T. repens*, including *T. uniflorum*. One accession of *T. uniflorum* had the lowest mean gall index, but only two plants classified as resistant, so it was not considered to be as resistant as *T. nigrescens* and *T. ambiguum*. This accession also had the smallest proportion of the root system affected by galls. The second *T. uniflorum* accession had no resistant plants.

Interspecific Hybrids

Interspecific hybrids between *T. repens* and *T. uniflorum* were first produced by Pandey (1957), and the

F_1 was successfully backcrossed to both parents. The F_1 was also self-compatible, in contrast to both parents, which are self-incompatible. The author interpreted this as indicating that the *S* gene of the two species occurs at different loci either on the same homologous chromosome or on non-homologous chromosomes. Studies on *T. repens* and *T. uniflorum* hybrids have also been published by Evans (1962a, b), Gibson et al. (1971), Gibson and Chen (1973), Chen and Gibson (1971b, 1972a, b), Pandey and Petterson (1978), Pandey et al. (1987) and Williams et al. (2006a).

Hybridization of these two species has had the same problems reported for other interspecific hybrids. For the first *T. uniflorum* × *T. repens* crosses produced by Pandey (1957), seed production was 30–50% that of intraspecific crosses, but germination (2 out of 30 seeds) and seedling survival (one of the two seedlings) were low. The two germinated seedlings also exhibited chlorophyll deficiencies. Pandey et al. (1987) subsequently found that the reciprocal cross – *T. repens* × *T. uniflorum* – was more successful. Although this combination produced no seed, rescued embryos ultimately produced plants that were viable. In contrast, *T. uniflorum* × *T. repens* F_1 plants had low germination, poor seedling survival and a high proportion of chlorotic or albino seedlings.

Evans (1962a) initially gained no seed from both *T. uniflorum* × *T. repens* and *T. repens* × *T. uniflorum* crosses, but observed development of embryos after hybridization of compatible genotypes. With the use of embryo rescue she then successfully produced several seedlings from each cross direction.

Gibson et al. (1971) also initially failed to produce hybrids from *T. repens* × *T. uniflorum* crosses, although pod enlargement and production of non-viable seed suggested that some embryo development was occurring. Further attempts, utilizing genotypic variation by concentrating on genotypes which exhibited pod enlargement, were successful.

Pre- and Post-fertilization Barriers

The findings of Evans (1962a), Gibson et al. (1971) and Pandey et al. (1987) showed the presence of barriers to hybridization between these two species. These have been studied in more depth by Evans (1962b), Chen and Gibson (1971b), and Chen and Gibson (1972a). As with interspecific crosses in general, pre-fertilization barriers appear to be less important than post-fertilization barriers. Evans (1962b) found *T. uniflorum* to have the longest pistil and style out of the ten species studied, and also the highest mean pollen tube growth rate. This growth rate was lower in *T. repens* × *T. uniflorum* crosses, but was still relatively good up to 24 h after pollination, unlike some other interspecific combinations where abnormal pollen tube growth was observed.

Conversely, Chen and Gibson (1972a) found *T. repens* x *T. uniflorum* had the slowest pollen tube growth with more abnormalities compared to other interspecific crosses, although few of these were the same as those studied by Evans. Pollen germination and fertilization was lower, but still occurred and fertilization was also slower than in *T. repens* intraspecific crosses.

Chen and Gibson (1971b) examined the seed development of *T. repens* × *T. uniflorum* crosses, and also observed delayed fertilization and a decrease in frequency of ovule fertilization. Abnormal growth of the hybrid endosperm appeared 4 days after pollination, followed by abnormal growth of the embryo. The authors speculated that failure of the embryos was due to starvation following the disintegration of the hybrid endosperm.

Embryo rescue has improved the success of interspecific hybridization between the two species (Evans 1962a; Pandey and Petterson 1978; Pandey et al. 1987).

Morphology of the Hybrids

Morphological descriptions of hybrids are generally intermediate to the two parental species. Pandey (1957) described the *T. uniflorum* × *T. repens* F_1 as vigorous and intermediate to the parents. Gibson et al. (1971) also described their *T. repens* × *T. uniflorum* F_1 hybrids as vigorous, with intermediate stipule shape and internode length. Example images of floral form were intermediate to the parents and the hybrids were stoloniferous perennials.

F_1, F_2 and BC_1's produced by Pandey et al. (1987) were generally intermediate, but variability was observed within the cross combinations, reflecting heterozygosity of the parents. Backcrosses to *T. repens* more closely resembled white clover in vegetative

characteristics, while those to *T. uniflorum* showed more *T. uniflorum*-like floral characteristics. Some plants showed genetic variation outside the range of either parent, notably the formation of taproot-like structures at the nodes. The authors describe most vigorous hybrids as having stronger and deeper root systems than *T. repens*, with most roots from the central crown. The hybrids were nodulated, suggesting that they are compatible with the same rhizobia as *T. repens*. Hybrids in the field were reported to have low vigor, but no further data has been presented on this.

Pandey and Petterson (1978) had previously reported similar transgressive segregation in a hybrid with a central taproot plus taproots at the nodes. A second hybrid had a root system more like that of *T. repens*.

T. uniflorum has been hybridized with 4*x* *T. occidentale* (Gibson et al. 1971; Gibson and Chen 1975), and the F_1 backcrossed to *T. repens*. In addition, *T. repens* × *T. occidentale* hybrids have also been backcrossed to *T. uniflorum*. Crossing *T. repens* and *T. uniflorum* with *T. occidentale* was used to overcome compatibility barriers between the two species. The multi-species hybrids were also seen as a means of introducing genes to *T. repens* from two species at once. *T. uniflorum* × *T. occidentale* has been backcrossed to *T. uniflorum*.

Scope for Improvement of White Clover

Despite the general difficulties of hybridization, this cross combination is relatively easy to achieve compared to some others (e.g., *T. ambiguum* × *T. repens*). Improvements to embryo rescue techniques have further improved the success of hybridization, and hybrids are also reasonably fertile (Pandey and Petterson 1978; Pandey et al. 1987). Good pairing of chromosomes and crossing over of genes between the two species occurs, indicating real potential for genetic exchange and the production of new variation. Most importantly, transgressive segregation has frequently been observed and there is also good potential to create new traits within white clover. Specifically, prospective improvements to the root characteristics of *T. repens* could dramatically increase drought tolerance, pest resistance and persistence, thus overcoming some of the limiting features of the species. This could extend its use on a regional and global basis and also have financial benefits to agriculture through decreased costs (irrigation, agrichemicals, re-seeding, fertilizers), and impacts on dry matter and animal production. A breeding program by the present authors aiming to achieve these objectives is currently underway in New Zealand. There are no known instances of the use of *T. uniflorum* as a crop in its own right. Individual plants are extremely small, and its chemical constitution is uncharacterized.

13.3.5 T. isthmocarpum *Brot.*

T. isthmocarpum (Moroccan clover) is an annual cross-pollinating diploid ($2n = 2x = 16$) Mediterranean herb found in the hills and fields of North Africa (Morocco, Algeria, Tunisia), Spain, Portugal, Corsica, Italy, Sicily and West Turkey (Zohary and Heller 1984). It has been identified in Australia as a potentially valuable species for spring production and as a waterlogging tolerant species suitable for a range of non-sandy soil types from loams to clays (Dear et al. 2003; Nichols et al. 2007). It has an efficient germination strategy involving brief hardseededness that breaks down rapidly in the field. This is associated with high temperature dormancy which protects it from germinating if it rains during summer (Norman et al. 1998). To date, however, no agricultural varieties have been commercialized. It has a unique karyotype among this group of species, it being the only diploid with two pairs of satellited subtelocentric chromosomes, although frequently only three satellites are visible (Chen and Gibson 1971a). Fertile hybrids with white clover were reported by Ferguson et al. (1990).

13.3.6 T. pallescens

T. pallescens is a diploid ($2n = 2x = 16$) perennial from high altitude Europe. It occurs on granite soils above 1,800 m altitude in the Pyrenees, Massif Central, and the Alps and mountains east of Bulgaria and Romania (Zohary and Heller 1984). In the central European Alps, it is an invasive pioneer species characteristic of recently de-glaciated moraines (Raffl et al. 2008). It is a highly self-fertile species which builds a large and persistent buried seed bank. It shows significant gene flow from valley to valley,

presumably by long distance seed head dispersal by wind (Raffl et al. 2008). Although it has some rooting from basal nodes, it shows limited vegetative spread. According to Chen and Gibson (1971a), its karyotype is indistinguishable from that of *T. ambiguum*. It has no agricultural role. Partially fertile hybrids with *T. ambiguum* were reported by Williams et al. (2006a). As shown in Table 13.1, there are very few accessions available in seed banks.

13.3.7 T. thalii *Vill.*

T. thalii is also an alpine species, not unlike *T. pallescens* in distribution, except that it is not found in Bulgaria or Romania and it is found in the mountains of Morocco (Zohary and Heller 1984). It is also a diploid, self-pollinating perennial, but has a different adaptation from *T. pallescens*, occurring in more stable alpine pastures rather than moraines, and being less invasive with lower seed production and larger seeds (Hilligardt 1993a, b). Its karyotype is similar to both *T. pallescens* and *T. ambiguum* (Chen and Gibson 1971a). Its role in agriculture is limited to its incidental natural occurrence in subalpine and alpine pastures. Williams et al. (2006a) reported that partially fertile hybrids with *T. ambiguum* could be made by embryo culture. Like *T. pallescens*, it has been little collected and more samples for seed banks should be obtained.

13.3.8 Other Species in Section Trifoliastrum

Other species in the white clover section that are not currently considered to have use in agriculture, or have not been adequately researched to contribute to the gene pool of white clover include the following.

13.3.8.1 *T. montanum* L.

T. montanum is a high altitude species from Europe and the Caucasus that is closely related to *T. ambiguum* (Ellison et al. 2006). It is diploid and genetically diverse (tetraploids have also been reported), occurring as three distinctive subspecies (Zohary and Heller 1984). It has not been evaluated as a potential agricultural species, but has been crossed with *T. ambiguum*, although the fertility of the hybrids was not given (Ferguson et al. 1990). If fertile hybrids are possible, then this species would fall within the tertiary gene pool of white clover.

13.3.8.2 *T. retusum* L., *T. suffocatum* L., *T. glomeratum* L. and *T. cernuum* Brot.

These diploid, annual species are widespread in the Mediterranean region and Eurasia (except *T. cernuum*, which is western only). All of them are relatively common weeds and gap-fillers in pastures in countries with temperate climates. To date, none has raised interest as a potential contributor to the gene pool of white clover.

13.3.8.3 *T. parnassi* Boiss. and Sprun

Ellison et al. (2006) confirmed the close phylogenetic relationship of *T. parnassi* with white clover. It is a diploid perennial species endemic to high mountain slopes in Greece (Zohary and Heller 1984). This species is of unknown potential as a source of traits for the improvement of white clover as there is little available germplasm in seed banks and there are few research reports. It is recommended that this species be collected and tested in interspecific hybrids with other species in section *Trifoliastrum*.

13.4 The Wild Relatives of Red Clover (*T. pratense*, Section *Trifolium*)

The closest wild relatives of *T. pratense* were listed by Cleveland (1985) on the basis of ease of crossing and chromosomal associations as: *T. diffusum* Ehrh. ($2n = 16$), *T. pallidum* Waldst. and Kit. ($2n = 16$), *T. medium* ($2n = 48$–80), including var. *sarosiense* (Hazsl.) Savul. and Rayss (*T. sarosiense* Haszl. ex Neileich) ($2n = 48$), *T. alpestre* L. ($2n = 16$), *T. heldreichianum* (Gibelli and Belli) Hausskn. ($2n = 16$), *T. rubens* L. ($2n = 16$) and *T. noricum* Wulf. ($2n = 16$). *T. andricum* Lassen ($2n = 16$) is also very close according to DNA sequence phylogenetics (Ellison et al. 2006). The DNA phylogeny also suggests that *T. squamosum* L., *T. barbeyi* Gibelli and

Belli and *T. lappaceum* L. (all $2n = 16$) are close relatives.

13.4.1 T. medium *and* T. sarosiense

Zig-zag clover, *T. medium* is a widespread rhizomatous perennial found from northern to southern Europe, and east ward to the Caucasus region and Iran. It occurs at diverse altitudes up to 2,000 m. Habitats range from forests and scrubland to meadows and it is also cultivated. It is very variable and has been loosely divided by Zohary and Heller (1984) into four varieties on the basis of flower structure and stem hairiness. However, these workers indicated that the intraspecies variation was far from resolved. It probably exists as a polyploid series, as chromosome numbers from $2n = 48$–126 have been reported. One such variant is *T. sarosiense*. This hexaploid ($2n = 6x = 48$) variety occurs at medium-high altitudes to 1,000 m in Romania (Zohary and Heller 1984) and has been used in hybridizations with *T. pratense* and other close relatives.

T. medium is of agricultural interest as a long-lived perennial clover suitable for grazed pasture (Townsend 1985). Several cultivars have been selected, but they have not met with commercial success, possibly because of a combination of very poor seed production, low forage yields and slow establishment of the species (Townsend 1985). In New Zealand, an experimental selection, G41, was produced by Rumball and Claydon (2005), but it never reached the market.

T. medium ($2n = 72$) and *T. sarosiense* ($2n = 48$) plants were found to be interfertile by Quesenberry and Taylor (1977), with only a slightly reduced seed set compared with intraspecific crosses. The progeny from *T. medium* as female were normal and as vigorous as either of the parent species. However, the reciprocal cross produced chlorotic plants, several of which did not flower. Progeny had $2n = 60$ chromosomes, as expected, except for two plants that were $2n = 58$. Pollen stainability of the hybrids was high (80–96%) and chromosome pairing between the genomes of *T. medium* and *T. sarosiense* also was high. Backcrosses to both parents and $F_1 \times F_1$ populations were produced, giving progeny that were largely normal, apart from a few chlorotic plants. Quesenberry and Taylor (1977) concluded that *T. sarosiense* was a distinct highly diploidized hexaploid species while *T. medium* was more complex with at least two chromosome races ($2n = 64$, $2n = 80$) and unstable intermediates, including the $2n = 72$ plants used in their study. *T. medium* × *T. sarosiense* hybrid germplasm was registered by Taylor and Quesenberry (1978).

There is significant interest in *T. medium* and *T. sarosiense* as potential sources of genes to improve the perenniality of red clover. However, the crosses have proved to be difficult. In a study of barriers to interspecific hybridization between red clover and *T. medium*, Repkova et al. (2006) found that pollen of *T. medium* ($2n = 56$) did not germinate on the styles of diploid red clover, and that the reciprocal cross behaved similarly. However, tetraploid red clover showed no such prefertilization barrier with *T. medium* in reciprocal crosses. Pollen from *T. sarosiense* ($2n = 48$) germinated in both $2x$ and $4x$ red clover crosses, but pollen from $2x$ red clover did not germinate on *T. sarosiense* styles. Successful germination of pollen from $4x$ red clover on *T. sarosiense* styles depended on the genotypic combination. The only crosses to develop hybrid embryos were those involving *T. pratense* ($4x$) × *T. medium*, where 160 crosses produced only four hybrid embryos.

Hybrids ($2n = 31$) between *T. sarosiense* ($2n = 48$) and diploid *T. pratense* ($2n = 14$) were produced by embryo rescue (Phillips et al. 1982) but these failed to produce viable pollen or seeds. The hybrids inherited the rhizomatous trait from *T. sarosiense* but the plants were not vigorous. Only one F_1 family produced normal flowers and no embryos from these reached maturity following pollination with *T. pratense*.

Hybrids between red clover and *T. medium* have been produced several times (Merker 1982; Sawai et al. 1990, 1995; Isobe et al. 2002), but to date, none have been commercially successful. Isobe et al. (2002), following Sawai et al. (1990, 1995), used tetraploid red clover and then doubled the chromosome number of the F_1 hybrids obtained by embryo rescue to achieve BC_1, BC_2, BC_3 and BC_4 progenies. This appears to have been the most successful project so-far but, even here, only weak expression of perenniality traits has been observed in the hybrids.

13.4.2 T. pallidum *and* T. diffusum

These are annual diploid species that are widespread in Europe and Eurasia. They have been crossed with red clover with varying but rather low success (Armstrong

and Cleveland 1970; Schwer and Cleveland 1972; Cleveland 1985) and all of the hybrids were annuals. The most fertile hybrids were obtained between 4*x* *T. pratense* and 4*x* *T. diffusum*. The annual nature of these species probably limits their value as genetic resources for extending the perenniality of red clover, but they may have a role in improving red clover seed production. Dabkeviciene et al. (2008) obtained higher numbers of flower heads in *T. pratense* × *T. diffusum* BC_1 hybrids to red clover and also observed some autogamy inherited from the *T. diffusum* parent.

13.4.3 T. noricum, T. alpestre, T. heldreichianum, T. rubens

These species are mainly alpine perennials that are of interest as potential sources of improved perenniality for red clover. However, although they have been hybridized among themselves, in various combinations (Quesenberry and Taylor 1976; Cleveland 1985), no successful hybrids with red clover have been reported to date. The DNA phylogeny of Ellison et al. (2006) places these species at a greater genetic distance from red clover than the annuals *T. diffusum* and *T. pallidum*. Phillips et al. (1992) obtained a single F_1 hybrid ($2n = 15$) using embryo rescue following *T. alpestre* ($2n = 16$) × *T. pratense* ($2n = 14$) crosses. The hybrid plant was both male- and female-sterile, and was morphologically more similar to *T. alpestre* below ground, having a creeping rhizome, while the leaves and flower heads were intermediate.

Quesenberry and Taylor (1978) successfully hybridized *T. sarosiense* with autotetraploid ($2n = 4x = 32$) *T. alpestre*, obtaining seeds and vigorous hybrid plants that were rhizomatous. These were partially fertile and produced low frequencies of F_2 and BC_1 seeds to both parents. However, crosses to both diploid and tetraploid *T. pratense* failed. Hybrid germplasm *T. sarosiense* × 4*x* *T. alpestre* was registered by Taylor and Quesenberry (1978). On DNA phylogenetic evidence (Ellison et al. 2006) *T. alpestre* appears to be very closely related to *T. medium*. Quesenberry and Taylor (1978) suggested that an ancestral form of *T. alpestre* may have been a diploid progenitor of *T. sarosiense*.

13.4.4 T. lappaceum *(Lappa Clover)*

Native to southern Mediterranean countries and Asia Minor, *T. lappaceum* is a diploid winter annual that also grows wild and is sown in the SE USA (Knight 1985). It is a close relative of red clover (Ellison et al. 2006). No breeding programs are known.

13.4.5 Recommendations for Future Actions

The lack of success in using interspecies hybridization to improve the perenniality of red clover indicates that new approaches are needed. One recommended approach would be to develop a new gene-pool based on the few available interspecific hybrids among red clover and its relatives, including the annual species. Although this may lead to the predominance of short-lived types in the early generations, provided that long-lived species are included, later generations may segregate fertile long-lived plants. This approach will require a long-term outlook by the breeder.

13.5 The Wild Relatives of *T. alexandrinum* (Egyptian Clover/Berseem)

Egyptian clover (berseem), *T. alexandrinum*, is a diploid ($2n = 16$) species widely grown as an annual feed crop in the Middle East, Mediterranean, Asia Minor regions and on the Indian subcontinent. A marked distinction has been made between forms with profuse basal branching, which have high agricultural value and forms with little or no basal branching (Zohary and Heller 1984). The species exhibits significant other genetic diversity, including that for duration of growth (number of cuts), yield and forage quality. However, needs have been identified for better dry matter yields in early cuts, a more extended vegetative growth period and resistance to root and stem rots (Malaviya et al. 2004b).

The closest relative of *T. alexandrinum*, according to protein profiles (Kumar et al. 2003), isozyme variation (Malaviya et al. 2004a) and DNA phylogenetics (Ellison et al. 2006) is *T. apertum*. *T. apertum* is an annual, diploid ($2n = 16$) meadow plant found in

Turkey and the Caucasus region, Italy and Greece. Indian varieties of *T. alexandrinum* did not cross naturally with *T. apertum*, but embryo rescue was successful in generating hybrids using *T. alexandrinum* as the female parent (Malaviya et al. 2004b). The F_1 hybrids were verified by isozyme characterization and were intermediate to the parents in morphology, although some showed better growth and branching than both the parents. Some of the hybrids were several weeks later in flowering than *T. alexandrinum* – a desirable trait that might extend vegetative yields. The hybrids showed pollen fertilities of 78–100% and bivalent formation at meiosis ranging from 89% to 98%. Seeds were obtained from some hybrids by selfing, and from others by backcrossing to *T. alexandrinum*. *T. apertum* offers potential for the expansion of the gene pool of *T. alexandrinum*.

13.6 Other Agricultural Species in Section *Trifolium*

13.6.1 T. hirtum *All. and* T. cherleri *L.*

Rose clover, *T. hirtum*, and cupped clover, *T. cherleri*, are extreme annual species widely distributed in fields and on roadsides throughout the Mediterranean region, and the Middle East and southwest Asia. They are a distinct, closely related pair on the basis of DNA sequences and a reduced chromosome complement of $2n = 10$ (Ellison et al. 2006). Rose clover has been developed commercially by selection of varieties suitable for Mediterranean climatic zones as far apart as Australia (Nichols et al. 2007) and California (Love 1985). Three cultivars of cupped clover were developed in Australia (Barnard 1972), but there has apparently been little recent interest. Because of their unique chromosome number, these species may be of limited value as a genetic resource for the improvement of any other species in the section. Attempted crosses of red clover with *T. cherleri* were unsuccessful (Quesenberry 1975).

13.6.2 T. affine *C. Presl and* T. arvense *L.*

T. affine and *T. arvense* are two closely related annual species in the red clover section. *T. arvense* ($2n = 14, 28$) is a self-fertilizing annual. It is particularly polymorphic and widespread throughout Europe, southwest Asia and the Mediterranean region, and has spread around the world in semi-arid habitats. *T. affine* ($2n = 12$) is a cross-fertilized annual with a much narrower distribution in Turkey and Bulgaria in dry habitats (Zohary and Heller 1984). Although both species can provide seasonal feed for grazing animals, the main interest is in their very high concentrations of foliar condensed tannins (CT). These are the only clover species with high foliar CT concentrations (Jones and Lyttleton 1971), and this has made them the target of some research efforts to identify the chemistry and genetics of CT production. To date, all efforts to transfer the high CT content to other species by hybridization have been unsuccessful.

13.6.3 T. incarnatum *L. (Crimson Clover)*

Crimson clover is well known for its large blood-red flower heads on erect stems, and is used in some regions, e.g. southern USA, as a roadside wild flower. It is a diploid ($2n = 14$) annual, native to southern Europe. It has received attention as a dual-purpose grazing and fodder species for farming in the USA (Knight 1985) and Western Australia (Nichols et al. 2007). It is soft-seeded, and produces well early in the season, but may lack persistence. Efforts to incorporate hard-seed are reviewed by Knight (1985).

13.6.4 T. dasyurum *C. Presl (Eastern Star Clover)*

This Mediterranean diploid annual species has been identified as a promising annual for Western Australia, and has been the subject of selection of a cultivar (Norman et al. 2005; Nichols et al. 2007). The selected material has a very specific and adaptive germination pattern, requiring summer heat followed by low diurnal fluctuating temperatures in autumn, and several weeks of high soil moisture before the seed will soften and imbibe. This provides considerable insurance that germination will not occur when rainfall is inadequate, so ensuring that a "false break" will not occur (Nichols et al. 2007).

13.6.5 T. purpureum *Loisel. (Purple Clover)*

This highly polymorphic Mediterranean diploid annual is widespread in its region of origin (Zohary and Heller 1984). It has been used in Australia as a winter annual pasture species since the 1950s. A variety "Paratta" was registered in 1971, and was developed to provide a low estrogenic replacement for subterranean clover on wet and waterlogged soils (Barnard 1972). Subsequently, another variety ("Electra") with disease resistance was registered in 2006 for high and very high rainfall zones (Nichols et al. 2007).

13.7 Section *Paramesus* (C. Presl) Endl

This section consists of just two species, *T. glanduliferum* Boiss. and *T. strictum* L., which have glandular hairs. DNA sequence phylogeny has placed it well apart from most other sections of the genus (Ellison et al. 2006). *T. glanduliferum* is of some agricultural interest.

T. glanduliferum originated in SE Europe, and is annual and diploid ($2n = 16$). It is adapted to sandy places among scrub and in fields. In Western Australia, it has been found to be well adapted to sandy loam to clay loam soils with a broad pH range. A variety was registered for Australia in 2001 (Nutt and Loi 2002). It has shown particularly good resistance to a range of pests (Nichols et al. 2007).

13.8 The African Clovers

Approximately 36 species of *Trifolium* are found in sub-Saharan Africa (Zohary and Heller 1984), in the central Eritreo-Arabian highlands, and extending to southern Africa as well. These species are all grouped into a new section *Vesicastrum* Ser. on the basis of DNA sequence phylogeny (Ellison et al. 2006). They were formerly classified into two sections – *Lotoidea* (33 species) and *Mystillus* (3 species) by Zohary and Heller (1984). The new section *Vesicastrum* also includes two groups of Mediterranean species, including six that were previously in section *Mystillus*, seven that were in section *Vesicaria* and four from section *Lotoidea*. These are discussed further in the next section.

The African group is relatively little studied. The students of systematics include Gillett (1952) and the Australia-based researchers Pritchard (1962) and Pritchard and t'Mannetje (1967). Vizintin et al. (2006) included five African species in a study of nuclear DNA content. These five species had the largest genome sizes in the genus (1C sizes: $2x$ *T. tembense* Fresen. 0.84 pg, $4x$ *T. africanum* Ser. 1.15 pg, $6x$ *T. burchellianum* Ser. 1.23 pg, $2x$ *T. rueppellianum* Fresen. 1.52 pg, $2x$ *T. semipilosum* Fresen. 1.96 pg).

13.8.1 T. semipilosum

Kenya white clover, *T. semipilosum*, is a stoloniferous perennial species from North and East Africa, especially Kenya, Uganda, Tanzania and Ethiopia. Formerly classified in series *Lotoidea* with white clover (Zohary and Heller 1984), it is now placed in section *Vesicastrum* on the basis of DNA phylogeny (Ellison et al. 2006), alongside all other African species and a few European species, including *T. hybridum* L., *T. michelianum* Savi, *T. ornithopodioides* L. and *T. fragiferum* L.

To date, it is the only African species that has been commercialized for agricultural use outside of Africa. *T. semipilosum* is a diploid ($2n = 16$), outcrossing perennial from very high altitude (1,400–3,200 m) grasslands and moist evergreen forests from north Ethiopia and Yemen south to Kenya, Uganda and Tanzania (Zohary and Heller 1984). It occurs as several distinct forms, e.g., var. *semipilosum*, which is hairy and adapted to slightly drier climates and var. *glabrescens* J.B. Gillett, which is almost glabrous and adapted to moist upland grasslands, often in association with kikuyu grass. Two further varieties have been identified in Ethiopia (Thulin 2008). Tetraploid ($4x = 32$) populations have also been reported. Cultivar "Safari" was released in 1973 after selection from Kenyan material of var. *glabrescens* by CSIRO researchers in tropical Australia. Agronomic studies of *T. semipilosum* are described by Hill (1989) and Ison et al. (1992) and flowering and seed-set problems by Ison and Parson (1992). One potential disadvantage is that the species has a requirement for very distinct and specific *Rhizobium* strains (Tesfaye and Holl 1998).

T. semipilosum is a potential source of a single gene resistance to clover root-knot nematode (*Meloidogyne trifoliophila*) in white clover. Genetic analyses of a segregating diploid population of *T. semipilosum* revealed that resistance to this parasitic nematode was inherited as a single dominant allele at a locus designated *TRKR* (Barrett et al. 2005). Using *T. repens* simple sequence repeat (SSR) markers and bulk segregant analysis, these authors located the linkage group carrying the *TRKR* locus in *T. semipilosum* and demonstrated macro-synteny between this linkage group and linkage group D homoeologues of *T. repens*.

T. semipilosum has been successfully crossed with other African species, including *T. masaiense* J.B.Gillett ($2x = 16$), *T. pseudostriatum* Baker, f. ($2x = 16$) and *T. rueppellianum* Fresen. ($2x = 16$), indicating close relationships among these species (Pritchard and t'Mannetje 1967). In all cases, shriveled seeds were obtained but apparently they were not germinated. *T. masaiense* and *T. pseudostriatum*, and *T. masaiense* and *T. rueppellianum* var. *rueppellianum* were also interfertile. According to DNA sequence data, *T. pseudostriatum* and *T. rueppellianum* are some distance further away from *T. semipilosum* than *T. masaiense*, which is very close (Ellison et al. 2006). There are many other African species (and some European species) closer to *T. semipilosum* than these, so further hybridization research with the African and European species might reveal the existence of species complexes that are, as yet, undetected. White and Williams (1976) found that fertilization occurred when *T. semipilosum* var. *semipilosum* was pollinated with white clover (*T. repens*). Globular embryos were formed and arrest of endosperm development may have been the main cause of failure. These authors detected genotypic variation for pod development and ovule enlargement, and suggested that a search for more compatible genotype combinations might be fruitful.

13.8.2 *T. burchellianum and T. africanum*

These perennial, outcrossing (self-incompatible) species occur in southern Africa. *T. burchellianum* ssp. *johnstonii* (Oliv.) J.B. Gillett ($2n = 12x = 96$) occurs further north from Tanzania to Ethiopia. *T. burchellianum* ssp. *burchellianum* ($2n = 6x = 48$) occurs at high altitudes in southern Africa only. By contrast, *T. africanum* ($2n = 4x = 32$) occurs across a range of altitudes and habitats in southern Africa (Zohary and Heller 1984). These polyploid species are related to a large group of diploid species that presumably includes the parental relatives (Ellison et al. 2006). Considerable scope exists to unravel the species relationships among the African species, especially given their closeness to some European species.

13.9 Mediterranean Clovers with African Affinities

The former section *Vesicaria* (Zohary and Heller 1984) consisted of seven Eurasian species that have more recently been re-classified with the African clovers on the basis of DNA sequence phylogenies (Ellison et al. 2006). These species have a very characteristic vesicular calyx that serves as a dispersal organ. They include *T. fragiferum* (strawberry clover) and *T. resupinatum* L. that are used in agriculture.

A second group of Mediterranean and Eurasian species with DNA sequence affinities to African species (Ellison et al. 2006) are six species that were previously classified with three African species in section *Mistyllus* (Zohary and Heller 1984). These include *T. spumosum* L. and *T. vesiculosum* Savi, which are of agricultural interest and *T. argutum* Sol., which has been reported to hybridize with white clover and its close relatives (Kazimierski et al. 1972).

A third group of Eurasian species with African affinities on the basis of DNA phylogeny (Ellison et al. 2006) are *T. hybridum*, *T. ornithopodioides*, and *T. michelianum*. These species were in the former section *Lotoidea* (Zohary and Heller 1984). This group includes two species of agricultural value.

13.9.1 *T. fragiferum (Strawberry Clover)*

This Mediterranean and central European diploid ($2n = 16$) perennial is adapted to wet saline or alkaline soils. Consequently, it has been spread widely by

man (Forde et al. 1981) and is found throughout much of north and west North America, parts of Australia, New Zealand and South America. Zohary and Heller (1984) identified five varieties. It is a prostrate plant with horizontal stems that root at the nodes (Gillett 1985; Townsend 1985), and consequently, is well adapted to close grazing. It is predominantly self-incompatible and cross-pollinated, although Mediterranean populations tend to be self-compatible (Davies and Young 1966). A number of cultivars have been commercialized, including "Palestine" and "O'Connors" (Australia), "Salina" and "Fresa" (USA, Townsend 1985). No hybrids with other species have been developed. Hybrids with *T. neglectum* C.A. Mey. were reported by Kazimierski et al. (1972), but this is a variety of *T. fragiferum* (Zohary and Heller 1984).

13.9.2 T. resupinatum *L. (Persian Clover, Shaftal)*

Persian clover is a widespread species in Southwest Asia, southern and central Europe, and the Mediterranean region. A distinctive cultivated form (var. *majus* Boiss.) that is not found in the wild has developed in Iran and neighboring countries (Zohary and Heller 1984; Gillett 1985). Both var. *majus* and var. *resupinatum* types have been developed for agricultural use, especially on winter waterlogged soils in Australia (Nichols et al. 2007), and in South Central USA, and many temperate countries (Gillett 1985).

13.9.3 T. spumosum *(Bladder Clover)*

This diploid ($2n = 16$) annual occurs throughout the Mediterranean region and is adapted to cool, moist winters and hot, dry summers. It has been found to be very productive in Western Australia on acid loams and clays in the 350–700 mm rainfall zone (Loi et al. 2003). It maintains a soil bank of hard seeds that soften in autumn in time for the wet season (Ghamkhar et al. 2007). A germplasm collection of about 400 accessions kept at the Australian *Trifolium* Genetic Resource Centre in Perth, Western Australia has been comprehensively characterized using eco-geographic data relating to the collection sites (Ghamkhar et al. 2007, 2008).

13.9.4 T. vesiculosum *(Arrowleaf Clover)*

This is a diploid ($2n = 16$), self-incompatible, deeply rooted, annual species, native to southern and Southeast Europe. It has gained some attention as a winter annual forage plant, especially in the southern USA (Miller and Wells 1985) and in Western Australia (Nichols et al. 2007). In Western Australia, two cultivars have been selected as fodder and grazing plants on moderately acidic light soils in medium-high and high rainfall zones.

13.9.5 T. argutum *(Syn.* T. xerocephalum *Fenzl.)*

T. argutum is a Mediterranean diploid ($2n = 16$) annual that has been reported to hybridize with white clover and its relatives, *T. isthmocarpum*, and *T. nigrescens* (Kazimierski et al. 1972). This report is unverified, but if correct, it would greatly widen the potential gene pool of white clover. However, large genetic distances between white clover and *T. argutum* were indicated by the DNA analyses of Ellison et al. (2006), suggesting that hybridization between these species would be unlikely.

13.9.6 T. hybridum *(Alsike Clover)*

Alsike clover, *T. hybridum*, is a perennial diploid occurring in fields and pastures, and on roadsides and river banks in the Mediterranean region as Far East as the Caucasus (Zohary and Heller 1984). It is widely cultivated as a fodder species in most temperate countries, and many cultivars exist (Townsend 1985).

As indicated above, and despite a similar floral morphology, it has been placed at a distance from white clover by DNA sequence phylogeny. Its nuclear 1C-genome size is 0.62 pg (Vizintin et al. 2006), and

the karyotype differs from that of white clover and its other near relatives in having satellited chromosomes that are the largest in the genome. In addition, the satellites are as long as the long arms of some other species (Chen and Gibson 1971a). Tetraploid *T. hybridum* was crossed with white clover using embryo culture, but the hybrid plants were predominantly chlorotic or albino. One survived to flowering, and a single green backcross seedling to white clover was obtained (Przywara et al. 1989).

13.9.7 T. michelianum *(Balansa Clover)*

T. michelianum is very closely related to *T. hybridum* according to DNA sequence comparisons (Ellison et al. 2006), but is an annual diploid species naturally distributed in the countries surrounding the Mediterranean Sea, from Portugal to Turkey (Zohary and Heller 1984). It is outcrossing and genetically diverse (Craig and Ballard 2000). Its genome size (1C = 0.654) is similar to *T. hybridum* (Vizintin et al. 2006), and its karyotype is also similar to that of *T. hybridum* (Chen and Gibson 1971a). It has been divided into two varieties, var. *michelianum* and var. *balansae* (Boiss.) Azn. It is used in more than 1.5 million hectares in Australian agriculture as an annual fodder species known as balansa clover (Craig and Ballard 2000). It is resistant to waterlogging and has moderate salinity tolerance (Rogers and Noble 1991; Rogers and West 1993). Several cultivars have been developed, including "Paradana," "Frontier," and "Bolta." It has good seed production, and the cultivars cause no animal fertility problems from phytoestrogens (Craig and Ballard 2000).

13.10 The American Clovers (Section *Involucrarium* Hooker)

Approximately 70 (over one fourth) of all *Trifolium* species occur in North and South America, and these form a monophyletic group (section *Involucrarium*) with no overlap with Eurasian species (Ellison et al. 2006). Most species occur in the western states, with California having 40 native species, including 12 endemic species (Crampton 1985). Thirteen species occur in South America, four endemic in Chile, and the rest mainly common to North America (Zohary and Heller 1984). From an agricultural perspective, no American species have been domesticated and, based on DNA sequence phylogeny, none appear to be closely related to any agricultural species from Eurasia. However, several have value as rangeland species for grazing in the west of North America (Crampton 1985). These include the rhizomatous perennials, *T. longipes* Nutt. in Torr. and Gray. and *T. wormskioldii* Lehm., which occur as polyploid series with relatively broad ecological ranges, and good grazing tolerance. Several western annual species also have grazing value in the Great Valley of California, where 65% of grazing animals in California are farmed (Crampton 1985). *T. stoloniferum* Muhlenberg is a tetraploid eastern North American species that resembles white clover but has never been domesticated or used in breeding. The South American species, *T. argentinense* Speg. and *T. polymorphum* Poir. ex Lam. and Poir. are perennial, stoloniferous, amphicarpic species occurring in the Campos region (Uruguay, South Brazil, East Argentina) where they are grazed as part of the native grassland vegetation and also sold as improved seed. This species has considerable potential for further plant breeding (Real et al. 2007). *T. polymorphum* ($2n = 16, 32$) (Zohary and Heller 1984; Vizintin et al. 2006) has a 1C DNA content of 1.025 pg (Vizintin et al. 2006). It has a unique amphicarpic breeding system, with self-fertilizing flowers under-ground, and allogamous, self-incompatible flowers requiring pollinators above-ground (Real et al. 2007).

13.11 Section *Trichocephalum* Koch

This is a group of nine annual species with specialized seed dispersal mechanisms, native to the Mediterranean region. It has one agricultural species, *T. subterraneum* L. This group of species is cytogenetically complex, with chromosome numbers ranging from $2n = 16$ (*T. subterraneum*) through $2n = 14$ (*T. pilulare* Boiss., *T. eriosphaerum* Boiss., *T. meduseum* Bl. ex Boiss.) to $2n = 12$ (*T. israeliticum* D. Zoh. and

Katzn.). This is likely to be an aneuploid reduction series from a $2n = 16$ ancestral form. Significant karyotypic polymorphism also occurs among the varieties of $2n = 16$ *T. subterraneum*, with chromosome rearrangements associated with reproductive isolation (Morley et al. 1956; Katznelson and Morley 1965).

13.11.1 T. subterraneum L. (Subterranean Clover)

T. subterraneum is a highly variable diploid annual species that has aerial flower heads that twist into the soil after fertilization, so that the seed matures underground. It occurs throughout the Mediterranean region and into England and S. Russia. Zohary and Heller (1984) have designated eight varieties, of which three have come into agricultural use. Var. *subterraneum* is the common form, best adapted to free-draining loams and sands, while var. *yanninicum* (Katzn. and Morley) Zohary is adapted to winter waterlogged soil types, and var. *brachycalycinum* (Katzn. and Morley) Zohary to neutral-alkaline soils. This species has been domesticated mainly by Australian researchers, who have selected within wild collections for genotypes of suitable adaptation, low estrogen content, and improved pest resistance for a wide range of environments (Collins and Gladstones 1984; McGuire 1985; Nichols et al. 2007).

Despite evidence for reproductive isolation due to chromosome rearrangements in this group, some interspecific hybrids have been produced. Katznelson (1967) obtained flowering specimens of *T. eriosphaerum* ($2n = 14$) × *T. subterraneum* var *brachycalycinum* ($2n = 16$) and *T. pilulare* ($2n = 14$) × *T. subterraneum* var. *yanninicum*. He also obtained hybrid plants of *T. eriosphaerum* × *T. israeliticum* and *T. pilulare* × *T. subterraneum* var. *subterraneum*, but both died before flowering. To date, these related species have not been used as a secondary gene pool for subterranean clover, but in future, they may be used for this purpose. Hybrids among the varieties of *T. subterraneum* have received some attention and deserve more. Reed et al. (1985) drew attention to the likely superior performance in Victoria, Australia of var. *yanninicum* × var. *subterraneum* hybrids.

13.12 Subgenus *Chronosemium* (Ser.) Reichenb.

This subgenus of 20 Mediterranean species is a divergent group of species, but clearly belongs to *Trifolium*. Although several species have pale purple or pink flowers, the majority have yellow flowers. It was separated from subgenus *Trifolium* (i.e. the remainder of the genus) by Ellison et al. (2006). This subgenus separated into two separate groups of species, corresponding with different chromosome numbers – a set of five species with $2n = 14$ and the remainder with predominantly $2n = 16$ – and one allotetraploid (*T. dubium* Sibth.) ($2n = 4x = 30$). Only two of the species are perennial – *T. badium* Schreb. and *T. rytidosemium* Boiss. and Hohen., both $2n = 14$. *T. dubium* has been shown by DNA sequence and genomic in situ hybridization (GISH) evidence to be an allotetraploid hybrid between *T. campestre* Schreb. ($2n = 14$) and *T. micranthum* Viv. ($2n = 16$) (Ansari et al. 2007).

None of the species of this subgenus have been commercialized for use in agriculture. However, the annual tetraploid species, *T. dubium*, has spread to pastures and meadows in many parts of the world, where it contributes seasonal animal feed, especially in spring, before seeding and dying. Because it is a clearly divergent subgroup (Ellison et al. 2006), it is unlikely that any species of this subgenus would provide genetic resources for hybridization with any of the species in the rest of the genus.

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Chapter 14
Vicia

John A. Bryant and Stephen G. Hughes

14.1 Introduction

14.1.1 Taxonomy and Geography

Vicia is an important genus within the family Leguminosae (also known as Papilionaceae or Fabaceae; Figs. 14.1 and 14.2). The family is very species-rich, containing over 9% of all known eudicot species (Doyle and Luckow 2003). *Vicia* is the type genus of the "vicioid" clade (Hanelt and Mettin 1989), which in turn is part of a higher level taxon (the IRLC) of legumes that lack the inverted repeat sequence in chloroplast DNA (Wojciechowski et al. 2004; Cronk et al. 2006). Within the vicioids, molecular phylogenetic analysis suggests a relatively recent divergence from the *Lathyrus* and *Pisum* lineage (Wojciechowski et al. 2000, 2004; Kenicer 2005). The genus is taxonomically complex and different types of phylogenetic analysis may give different patterns of interspecific relationship (see e.g., Potokina et al. 1999; van de Wouw et al. 2003a, b). There is also a good deal of genetic diversity within species (see e.g., Galasso et al. 1997; Huh and Huh 2001; Shiran and Raina 2001; van de Wouw et al. 2003a), which is obviously relevant to the possible utilization of wild *Vicia* species in agriculture.

There are about 140–150 *Vicia* species[1] (Kupicha 1976) although some authorities suggest that the total may be around 200 (see Hanelt and Mettin 1989). Several new species have been described in the last two decades (Erik and Demirkus 1998; Maxted et al. 1991) and it is likely that more remain to be discovered in the more inaccessible regions of the genus's range. Some taxa are hard to define and several species exist as multiple subspecies or races (van de Wouw et al. 2001; USDA-GRIN) while others are regarded as aggregates or complexes (Bennett and Maxted 1997; van de Wouw et al. 2001, 2003b). Thus, in *V. sativa*, different lineages have different chromosome numbers (see Sect. 14.1.3), while the genetic differences between two particular biotypes of *V. benghalensis* are large enough to prevent cross-breeding between the two (Galasso et al. 1997). The existence of many well-defined races or biotypes, which may or may not be defined as separate species, can be taken as an indication of rapid evolution. Specific evidence for this comes from studies of restriction fragment length polymorphisms (RFLPs) in the *Vicia sativa* complex (Shiran and Raina 2001).

Vicia is a mainly Euro-Asiatic genus with a major center of diversity in the eastern Mediterranean or western Asiatic region. There are also 27 New World species known, giving minor centers of diversity in North and South America (reviewed by Endo et al. 2008). Morphological and molecular phylogenetic analyses nest the New World *Vicia* well within the Old World species indicating a relatively recent divergence of the two groups (Endo et al. 2008). It is likely that the genus's genetic diversity in the New World arose from three separate invasions via the Tertiary land bridge that linked the American continent with Eurasia (Tiffney 1985; Doyle and Luckow 2003). Certainly today, molecular phylogeny, based on sequences of the internal transcribed spacer (ITS) in the genes encoding ribosomal RNA, identifies three

[1]Readers should be aware that some Web sites present very inaccurate estimates of *Vicia* species numbers, including one that gave a total of nearly 900

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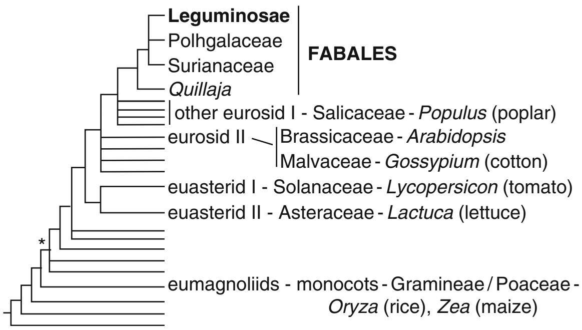


Fig. 14.1 Position of the Leguminosae/Fabaceae amongst the angiosperms (Doyle and Luckow 2003; Soltis et al. 2000)

distinct groups of New World *Vicia* (Endo et al. 2008; Fig. 14.3).

14.1.2 General Features

The genus consists mainly of a mix of annual and perennial species (with only a few biennials); most are climbing or scrambling herbaceous plants with tendrils. However, the main domesticated species, *V. faba* does not exhibit the climbing habit and lacks tendrils. All members of the genus possess the characteristic "papilionaceous" (i.e. "butterfly-shaped") flowers like those on the pea plant (Fig. 14.4). The flowers are borne in racemes each of which may contain between 1 and 40 individual flowers, according to the species. In many species, the flowers are brightly colored and strikingly beautiful. The flowers produce nectar and are mostly pollinated by bees of various species. Seeds are borne in two-valved dehiscent pods with several seeds in each pod.

14.1.3 Genomes

The majority of *Vicia* species have a diploid chromosome number of 14, but there are species in which $2n = 12$ and $2n = 10$. Many of the species with $2n = 10$ and $2n = 12$ are in the Hypechusa section of the genus (Caputo et al. 2006). Tetraploid species are known (e.g., *V. cracca*, $2n = 28$) as are species with variable karyotypes. Thus, within the *V. sativa* aggregate, there are lines with 10, 12 and 14 chromosomes while in *V. amphicarpa*, strains with $2n = 10$ and $2n = 14$ have been identified (Raina et al. 2001). Haploid genome sizes vary between ca 1,175 Mbp and ca 13,255 Mbp (Bennett and Smith 1976; Bennett and Leitch 1997; Bennett et al. 2000; Caputo et al. 2006). There is no apparent correlation with chromosome numbers but there is some correlation with life-style: perennial species tend to have larger genomes than annual species (Bennett and Smith 1976; Bennett and Leitch 1997; Bennett et al. 2000).

Variations in genome sizes arise almost entirely from variations in repetitive DNA sequences, including genes encoding rRNA, "satellite" DNA, simple-sequence repeats and retrotransposons (Bryant 1976; Pearce et al. 1996; Hill et al. 2005; Neumann et al. 2006). The latter comprise between 20 and 45% of the genomes in different species. Several classes of retrotransposons have been identified, including LINEs (which form a very complex and heterogeneous group of sequences), Ty1-copia and Ty3-gypsy elements, including the giant (22–25 kbp) retroelement *Ogre*. Comparisons between *V. faba*, *V. melanops* and *V. sativa* show that the Ty3-gypsy elements are very abundant and comprise18–35% of the genomes of these three species. Ty1-copia group elements are less numerous, while LINE elements are the least abundant retrotransposons. At the other end of the range, the *Ogre* element exhibits hugely variable copy numbers between species (Neumann et al. 2006). For example, in *V. pannonica* (1C = 6,615 Mbp), there are 1×10^5 copies, comprising 38% of the genome while in *V. faba* (1C = 13,255 Mbp), there are between 100 and 500 copies, comprising on average 0.1% of the genome.

14.1.4 Agricultural Status

The utilization in agriculture of *Vicia* species has been limited by the widespread occurrence of toxins (antinutritional factors) in this genus. These toxins include lectins, nonprotein amino acids, protease inhibitors, cyanogenic compounds and condensed tannins (see Sect. 14.6). The selective advantage of producing these compounds is clear: they protect against herbivory. Nevertheless, their presence is a problem for the nutrition of humans and other monogastric animals. Ruminants are much less affected by some of the toxins and so some *Vicia* species, which cannot

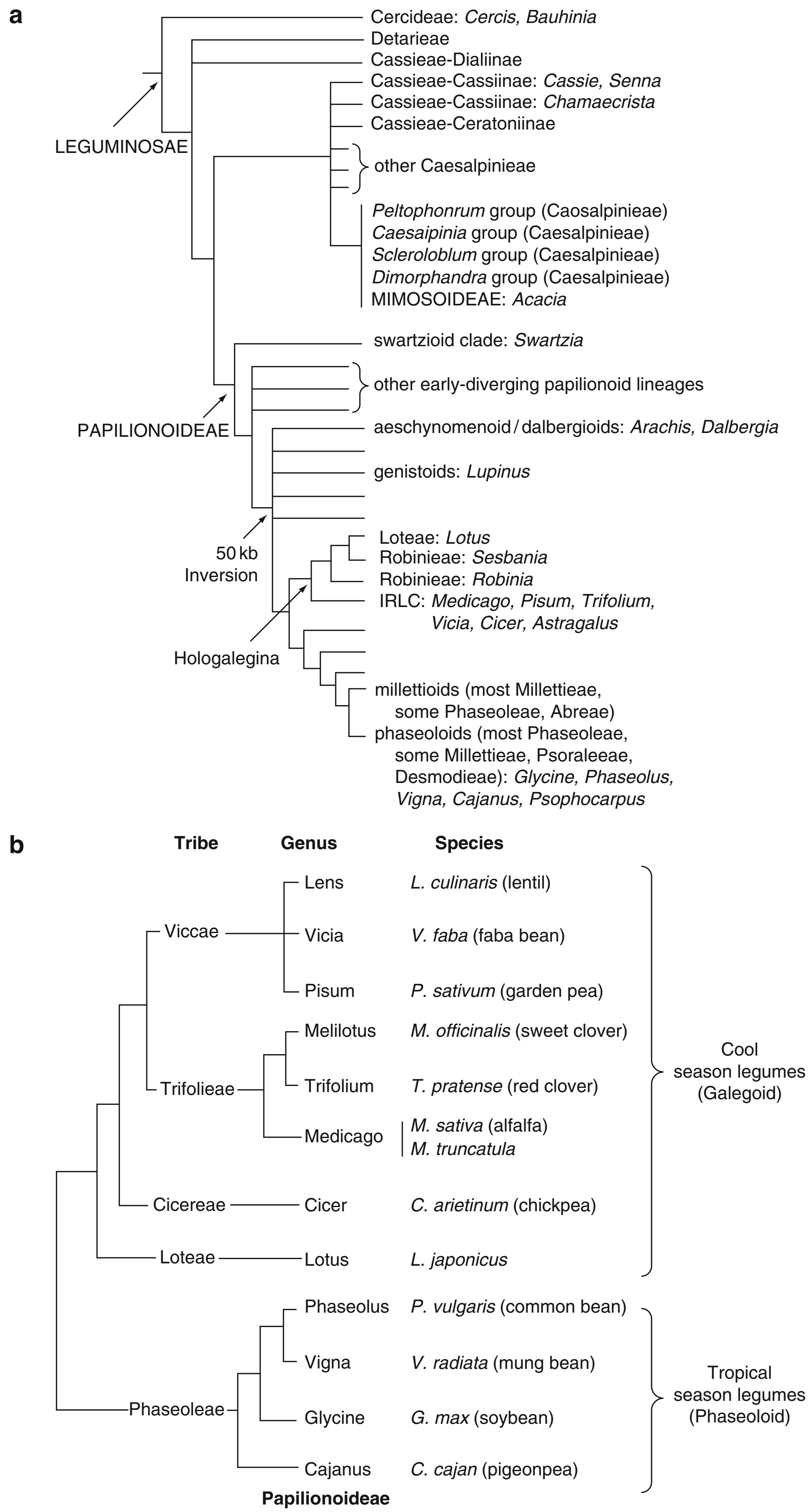


Fig. 14.2 (**a**) Position of *Vicia* and other IRLC genera within the Leguminosae. (**b**) Position of *Vicia* within the Papilionoideae. From Choi et al. (2004)

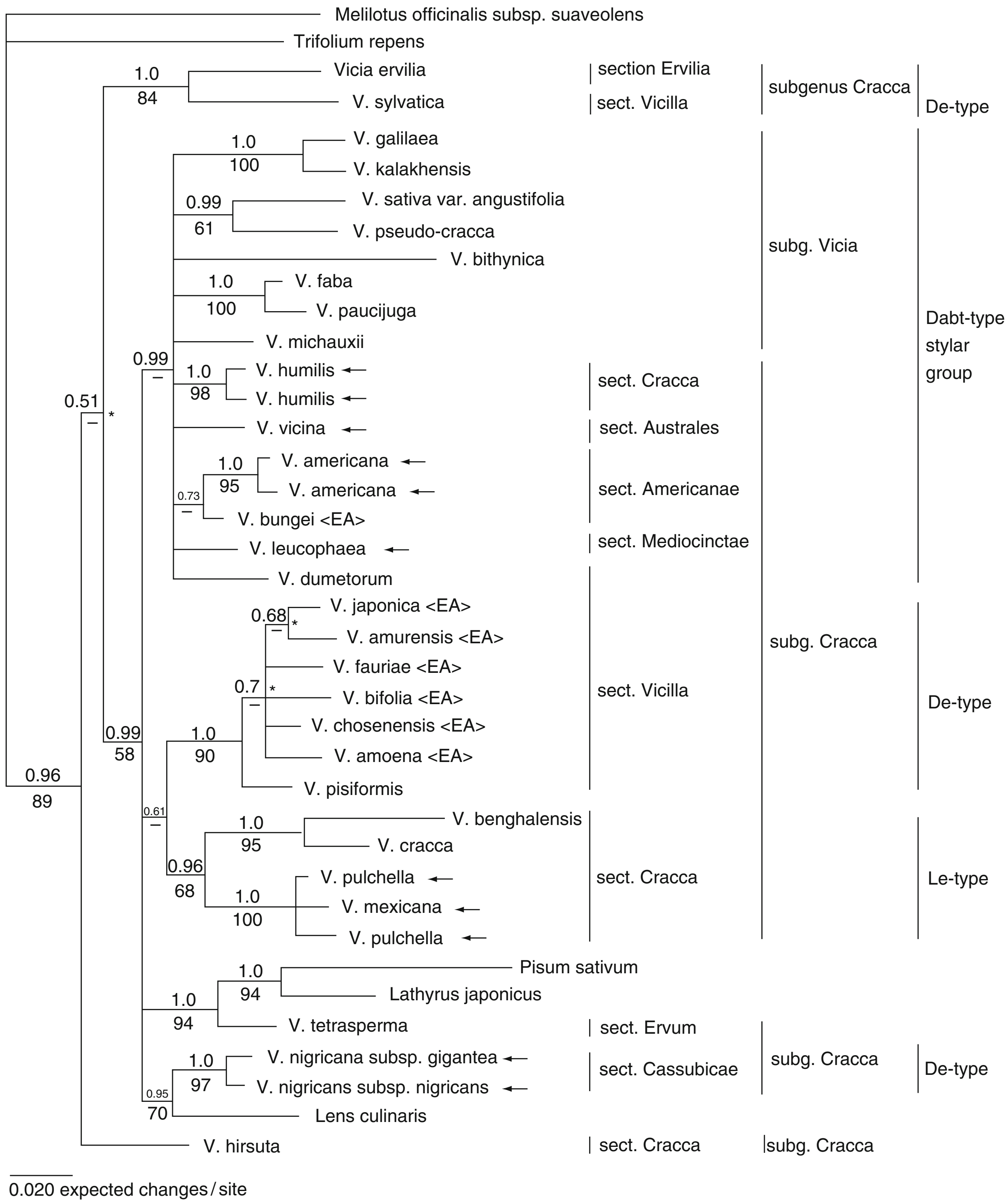


Fig. 14.3 Maximum likelihood tree based on ITS sequence data for representative New World *Vicia* species (indicated by *arrows*). From Endo et al. (2008)



Fig. 14.4 Flower and immature seed pods of *Vicia sativa*. © Colin Duke

be used to feed humans (or pigs), are acceptable for feeding cattle.

Thus, relatively few *Vicia* species have been used during the millennia of human agriculture. Today, only one of these, *V. faba* ("broad bean") (Fig. 14.5) is very widely grown for human nutrition. Based on findings in North-west Syria, its use goes back to at least the tenth millennium BP (Tanno and Willcox 2006; see also Sect. 14.3). However, despite this long history, including selection for low toxin content, this species can still cause "favism" in people with a genetic deficiency in glucose-6-phosphate dehydrogenase.

Other species that have in the past been domesticated, or cultivated without domestication or gathered from the wild include *V. sativa* (common vetch), *V. ervilia* (bitter vetch), *V. peregrina* (rambling vetch) and *V. narbonensis* (narbon bean). It has been suggested that some instances of human use were accidental in that seeds of wild *Vicia* species became mixed with those of other pulses that already had a place in human agriculture (Erskine et al. 1994) or those plants were harvested with grasses when animal feed was gathered (Melamed et al. 2008). Whatever the means of incorporation into human or animal food, the presence of toxins was a problem; it is likely that in both human and animal nutrition, *Vicia* seeds were "diluted" with less poisonous pulses and with cereals. Cooking also removes some of the *Vicia* toxins and there is evidence from one Neolithic site that seeds of *V. peregrina* were cooked (Melamed et al. 2008).

Another species that has been cultivated is *V. articulata*, the single-flowered vetch. Laghetti et al. (2000) state that this has been used for green manure, forage and even human food. It is still grown (but not for human food) in southern Europe (mainly Spain), western Asia and to a much lesser extent in Australia. In Italy, the country in which these authors have a particular interest, records suggest that it was grown until the 1950s but that its use was then discontinued.



Fig. 14.5 Flowers of *Vicia faba*. Photograph by Ken Redshaw © University of Leeds

Over the long period of human agriculture, *Vicia* species other than *V. faba* and *V. narbonensis* were mostly discarded from human diets, except *in extremis*, and were "demoted" to be used only in animal food (but see Sect. 14.3). Nevertheless, it is widely held that the genus *Vicia* has great potential for human and animal nutrition and as green manure (Enneking and Tate 2007).

14.2 Conservation

A search of the 2009 IUCN Red List does not bring up any *Vicia* species as threatened with overall extinction (*http://www.iucnredlist.org/*). However, some species

are rare (see e.g., Castiglione et al. 2007) because they are confined to particular habitats within particular regions. This type of situation raises concerns about conservation at a more local level. Thus, many Old World *Vicia* species have a preference for the types of soil that are widely found in the alluvial plains of the eastern Mediterranean region (Tanno and Willcox 2006). In the major center of diversity, many examples of these habitats have become degraded by human activity. In 1995, seven wild *Vicia* species were threatened with local extinction in this region, while a more recent survey in Syria suggested that several species are under threat (Keiša et al. 2008). For many regions local databases are available in which the locally threatened or vulnerable species are listed (e.g., for Wales, UK: *http://www.watsonia.org.uk/WBull77a5.pdf*). Most of these indicate some level of threat to at least one, and often more, species. Reduction and fragmentation of populations mean that for the vulnerable species, genetic erosion is a significant threat, made the more significant by the increased interest in the use of several *Vicia* species as forage crops (see next section).

Conservation is important in the general context of maintaining biodiversity and of preserving genetic resources for new breeding programs. Many *Vicia* species are conserved as germplasm. The major germplasm resource for *Vicia* species with actual or potential economic importance is at ICARDA, Aleppo, Syria (*http://www.icarda.org*). Other important collections are at the Vavilov Institute of Plant Industry, St. Petersburg (*http://www.vir.nw.ru*), the Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben (*http://www.ipk-gatersleben.de*) and the Millennium Seed Bank at Wakehurst Place, UK (part of the Royal Botanic Gardens, Kew: *http://www.kew.org/msbp*). There are smaller collections at other centres, e.g. at the Institute for Agrobotany, Tápiószele, Hungary there is a collection of European wild species which are related to past and present crop species (*http://www.rcat.hu*; Holly et al. 2005).

Maxted, the leading expert on this genus, along with his colleagues, emphasizes the importance of understanding in depth the ecogeography of individual species (e.g., Bennett et al. 1998; Maxted 1999). Conservation in situ may then be used as a complementary technique for germplasm preservation in an overall conservation strategy (e.g., Maxted et al. 1997; Maxted 2003).

14.3 Origin, Evaluation and Selection of Allied Crop Plants

In this particular genus, it is often hard to make a distinction between wild species and species used as crops. There are species which appeared in the past to be gathered from the wild; others have been cultivated but remain essentially wild species while yet others have been subject to domestication and selection. Thus, of the relatively few *Vicia* species that have been adopted as crop plants, a number of these were, and still are gathered from the wild or are cultivated without becoming domesticated. This will be become clearer as we now discuss specific examples.

Based on archeological records, *V. ervilia* (Fig. 14.6) is regarded as one of the eight "founder crops" of human agriculture. Further evidence, albeit fragmentary, suggests that this domestication occurred only once (Zohary 1999) and presumably involved selection for desirable traits, including lowered toxicity. It has also been argued that *V. faba* should be included as one of the founder species following morphological and anatomical identification at Tell el-Kerkh in Northwest Syria (Tanno and Willcox



Fig. 14.6 *Vicia ervilia*. © D. Hassler. University of Karlsruhe

2006). As briefly mentioned in Sect. 14.1.4, this places the domestication of *V. faba* in the late tenth millennium BP, contemporary with the other founder species. Many different cultivars or races of *V. faba* are now in use in different regions of the world and several centers of agricultural selection have been identified.

The immediate progenitors of *V. faba* remain unknown. No closely related wild relative of *V. faba* has ever been identified and it does not hybridize with any modern wild *Vicia* species (Maxted et al. 1991; Muehlbauer et al. 1994). Indeed, it seems likely that the wild progenitor is now extinct (Tanno and Willcox 2006).

V. sativa (common vetch) has also been domesticated (Erskine et al. 1994; van de Wouw et al. 2003b). Erskine et al. (1994) suggest that this initially happened accidentally when *V. sativa* grew as a weed in stands of *Lens culinaris* (lentil). Selection led to less hard seeds, lower levels of seed dormancy, greater yield and greater competitiveness; eventually the common vetch was grown in single-crop stands as a forage crop. There is also evidence that it was used as green manure in pre-industrial agriculture in the UK; the organic farming movement is promoting a renewal of the practice (e.g., Garden Organic 2009). Nevertheless, it is often regarded as a weed rather than as a useful plant (e.g., Aarssen et al. 1986).

Evidence for earlier selection of desirable traits in *V. sativa* comes from recent research in western Turkey (Celiktas et al. 2007). Comparison of wild and cultivated populations in that region showed that all the wild plants had a $2n$ chromosome number of 10 whereas for the cultivated population, $2n = 12$. Further, the cultivated plants had bigger seeds with less hard seed coats and higher germinability than the wild plants, again giving evidence for selection during domestication. However, it is not clear whether the increase in diploid chromosome number arose as a consequence of selection of desired characters or whether the lineages with desired characters had the higher chromosome number. In Brazil, it has been suggested that a reduction in $2n$ chromosome number from 14 to 12 is a result of founder effect in terms of the lineages of *V. sativa* used as forage crops.

Another species that has undergone some degree of domestication is *V. narbonensis* (Bennett and Maxted 1997). In times past it had been regarded as the likely immediate ancestor of *V. faba* (Hopf 1973) but modern phylogenetic analyses show that this cannot be so: the two species are quite distantly related (van de Ven et al. 1993). *V. narbonensis* exhibits a good deal of diversity between different populations and a recent phylogenetic analysis using retrotransposon-based sequence-specific amplification polymorphisms (SSAP) suggests that at least some of this diversity arose deep in the history of the species (Sanz et al. 2007). Today in the Middle East it often occurs in grassland or as a weed in cultivated land (Bennett and Maxted 1997). However, it is still grown as a crop for animal feed and human nutrition in Syria, northern Iraq and parts of Turkey (Bennett and Maxted 1997) but in earlier centuries was cultivated much more widely in the eastern Mediterranean region. In this regard it resembles several other *Vicia* species, cultivation of which has become less widespread. However, *V. narbonensis* is undergoing a "renaissance." Its seeds, which are cooked before they are eaten, are described as being of high quality (but see Sect. 14.4); there are several selection breeding programs underway (see Sect. 14.4).

A relatively recent worldwide survey presented in the 2001 version of the ILDIS database (ILDIS 2001) lists 21 *Vicia* species that are used today as forage or fodder crops. Some of these have already been mentioned as being domesticated or at least harvested in the early centuries of human agriculture, namely *V. ervilia*,[2] *V. faba*, *V. narbonensis*, *V. peregrina* and *V. sativa*. For the other 16 species, it is presumed that their use is more recent and/or localized to particular areas, depending on the species. These include *V. villosa* in several countries, *V. pannonica* in Turkey, *V. ervilia* and *V. articulata* in Spain and *V. benghalensis* in Australia (Enneking and Tate 2007).

The same database lists five species as finding current use in human nutrition. These obviously include *V. faba* and *V. narbonensis* but the list also mentions *V. amurensis*, *V. hirsuta* and *V. unijuga*. It is worthwhile to examine the use of the "extra" three species. *V. amurensis* is native to eastern Asia, including Japan. According to Kunkel's comprehensive guide, *Plants for Human Consumption* (1984), it is the young leaves that are harvested from the wild. The leaves are cooked before being eaten. *V. hirsuta* is a native of western Asia, North and North-east

[2]One interesting modern use of *V. ervilia* is its inclusion in diets of poultry in order to induce molt (Mohammadi and Sadeghi 2009)



Fig. 14.7 *Vicia unijuga* (Photograph from http://www.botanic.jp)

Africa and Europe; it has been introduced into the USA; in Europe it is regarded as a weed. In Ethiopia it is gathered from the wild; the shoots are used as green vegetables and the seeds are roasted or boiled. In Europe and Asia, *V. hirsuta* has been used as human food under famine conditions but today its possible more widespread acceptance, both in western Europe and the USA, is being promoted by the "food-from-nature" movement (see e.g., Plants for a Future 2009). However, the seeds contain trypsin inhibitors and the toxic nonprotein amino acid canavanine; they must therefore be prepared and cooked properly prior to consumption. *V. unijuga* (Fig. 14.7) is confined to the temperate regions of Asia. Its shoots are used as a food in parts of China, but usually only under conditions of food shortage.

14.4 Wild Species: Potential and Possibilities for Use in Agriculture

14.4.1 Introduction

It is abundantly clear that the legumes, in general, have a much greater potential for human use than is currently utilized. Even for those species that are widely grown, research and breeding programs have lagged behind those on cereal crops (Graham and Vance 2003). Further, the newer molecular technologies have had almost no impact on legume breeding (Varshney et al. 2009). The result of this relative lack of effort is that increases in yield have been far smaller than those seen in cereals and indeed, in some countries, yields of leguminous crops have actually declined (Graham and Vance 2003). This lack of attention is strange, especially in view of the nitrogen-fixing capabilities of the legume–*Rhizobium* symbiosis, giving legumes the ability to succeed on N-poor soils as well as supporting their potential as interplanted or break crops.

What is true for legumes in general is also true for the genus *Vicia* in particular. It is true, as has already been stated, that over 20 wild *Vicia* species are already in use, mainly as animal feed; other uses include animal bedding, application as green manure or in pasture (thus exploiting the N-fixing ability) and for a small number of species, human nutrition. Secondly, we have seen that some of these species, typified by *V. sativa* and *V. narbonensis*, exist both as wild forms and as a series of domesticated and selected forms. The potential to extend the use in agriculture of this genus is in some senses thus already acknowledged. Nevertheless, the research aimed at exploiting this potential has been relatively insignificant. However, over the past two decades there has been growing pressure to start taking more seriously the need for focussed research, typified by the question "When and where will vetches have an impact as grain legumes?" (Francis et al. 2000; see also Tate and Enneking 2007a). This pressure has been translated into action in the form of a number of breeding programs for specific wild species and especially for *V. narbonensis* and *V. sativa*.

14.4.2 V. narbonensis

The "narbon bean," *V. narbonensis* is regarded as having potential for growth in areas where water supply is restricted; breeding for even better drought tolerance is under way in Australia (Siddique et al. 1996; Francis et al. 2000) and at ICARDA, Aleppo, Syria (Ahmed et al. 2000; Francis et al. 2000). Individual laboratories in the USA and Europe (e.g., at Gatersleben, Germany: Muntz et al. 1998; Rolletschek et al. 2004) are focussing on other aspects of crop growth, yield and quality (see below). In an early series of trials in western Australia, the accessions

used in breeding programs exhibited slower development than *V. faba*. However, the yields finally obtained were comparable to those obtained with *V. faba* (*V. faba* is better under wet conditions; *V. narbonensis* is better under dry conditions; Siddique et al. 1996).

In addition to enhanced drought tolerance, other improvements that are being sought through breeding programs include faster development (Siddique et al. 1999), higher yield of seeds (Francis et al. 2000), higher seed protein content (Rolletschek et al. 2004), higher content of essential amino acids in seed protein (Muntz et al. 1998) and greater resistance to diseases (Ahmed et al. 2000) and to parasitism by broomrapes, *Orobanche* spp. (Sillero et al. 2005; Nadal et al. 2007).

One of the advantages of narbon bean is its high content of sulfur amino acids, an important factor when considering the use of legumes in the diet. However, this is also a disadvantage because much of the cysteine occurs in the form of γ-L-glutamyl-S-ethenyl-L-cysteine, which gives a very unpleasant off-flavor. Royo et al. (2007) suggest strategies, both chemical and genetic, for dealing with this problem and conclude that the previously "much maligned and ill-regarded narbon bean" now has great potential.

14.4.3 V. sativa

Of all the "wild" *Vicia* species that have been partially domesticated, *V. sativa* (common vetch) is the most widely grown in modern times; it is used as a forage and fodder crop for ruminants, as green manure and as a component of hay. As may be expected from this wider usage, there has also been more research and a larger number of breeding programs than with most other *Vicia* species. The existence of different karyotypes has already been mentioned although it appears at present that all the cultivated lineages fall into the $2n = 12$ karyotype. A more general assessment of genetic variety within the *V. sativa* complex has been made by Potokina et al. (2002). They performed an amplified fragment length polymorphism (AFLP) fingerprint analysis of 673 accessions from the Vavilov Institute of Plant Industry (St. Petersburg) seed collection and 450 accessions from the worldwide collection at the Institut fur Pflanzengenetik und Kulturpflanzenforschung (Gatersleben).[3] Seventy clearly recognizable polymorphic fragments were generated which were all present, although to greatly varying extents, throughout the whole natural range. However, they also identified accessions which, despite very different geographical origins, had very similar amplified fragment length polymorphism (AFLP) patterns.

As yet there have been no attempts to use these AFLP fingerprints or other molecular markers in *V. sativa* breeding programs but this must surely emerge (see Sect. 14.5). Current breeding programs are mostly based on traditional methods of selection and are aimed at improving seed yield (Cakmakci et al. 2003, 2006; van de Wouw et al. 2003b), yield of biomass for use in hay (Mihailovic et al. 2005), cold-tolerance (van de Wouw et al. 2003b), resistance to fungal diseases (Ahmed et al. 2000), insect pests (van de Wouw et al. 2003b) and *Orobanche* parasites (Perez-de-Luque et al. 2006). Also, and very importantly if this species is to be used in nutrition of monogastric animals (including humans), there is keen interest (see Tate and Enneking 2007b) in reducing the anti-nutritional factors – toxins – that are synthesized by this and indeed many other *Vicia* species. In common vetch, the toxins include cyano-alanine, cyanogenic glycosides and the favism-inducing compounds, vicine and convicine. However, despite the efforts of several research groups (see Tate and Enneking 2007b), no toxin-free lines of *V. sativa* have yet been produced (see also Sect. 14.6).

14.4.4 Other Wild or Partly Domesticated Vicia *Species*

Of all the other wild *Vicia* species in which there are domesticated (or partly domesticated) populations only one appears to have a breeding program devoted to it. This is *V. articulata* (Laghetti et al. 2000) and the particular concern is to produce cultivars that do not accumulate the non-protein amino acid, canavanine and which have a lower content of condensed tannins (Vioque et al. 2008). For other species, such as

[3]Note that these authors did not make use of the germplasm collection of economically important *Vicia* at ICARDA, Aleppo, Syria

V. ervilia and *V. villosa*, some comparison of different lines has taken place (e.g. El Moneim 1993) but this does not appear to have led as yet to any concerted breeding programs.

14.5 Breeding Strategies

14.5.1 Introduction

It will be apparent by now that the genus *Vicia* represents a large range of genetic variation, most of which has not been utilized or even investigated. The focus on *V. faba* as the main agricultural species has been partly responsible for this. Because of inability to hybridize with any other *Vicia* species, it has not been feasible until very recently to consider introgression of traits from wild species into *V. faba*. However, recent advances in genetic modification and genomics may mean that the situation is about to change. This will lead to further research on wild species both because of possible use of particular traits in the breeding of *V. faba* and because of the wider uptake of wild species in agriculture.

14.5.2 Background Information

In the selection and breeding and the identification of useful traits in wild species, Bennett (1999) has emphasized the importance of properly curated and documented ex situ collections, including germplasm banks and herbaria. Knowledge of the provenance, including soil type, altitude, climate and specific habitat of a particular accession provides extensive background information on the general genetic traits possessed by that accession. To put it simply, the breeder would be foolish to look for drought tolerance in a species or in a population that grows in an area of high rainfall. Thus, breeders interested in growing grain legumes in semi-arid regions in Australia have made collections of wild species growing under similar conditions in South-east Europe and western Asia (e.g., Siddique and Loss 1996; Berger et al. 2002). In this context, Duc et al. (2009) discuss the complications that may be imposed by variable levels of allogamy in the maintenance and deployment of phenotypically or genotypically characterized accessions.

14.5.3 Hybridization

Hybridization followed by backcrossing has been a traditional means for introgression of traits from one accession or species into another. As already noted, wild *Vicia* species have not been used in this way in the breeding of *V. faba* because it will not hybridize with any other extant member of the genus. However, hybridizations between other *Vicia* species have been recorded (reviewed by Hanelt and Mettin 1989) although wide hybridizations between members of different tribes usually fail. Indeed, even some of the hybridizations within tribes lead to the production of infertile offspring. This may indicate relatively rapid sequence divergence between species, notwithstanding the general genetic synteny between different legume clades (see Sect. 14.5.4). Nevertheless, analysis of some of the tetraploid species gives evidence for interspecific hybridization followed by the formation of allotetraploids in the evolutionary history of the genus (Li et al. 2001).

A different approach may involve the use of synthetic hybrid populations which offer an alternative and potentially tractable route to achieving heterosis where F_1 hybrid seed breeding is not possible. Both spontaneous outcrossing and deliberate assembly of mixed intercrossing populations offer the possibility of enriching heterozygosity and releasing heterosis (Link 1990). It has been suggested that considerable heterotic performance benefits may be obtained for *V. faba* through this strategy (Link et al. 1994).

14.5.4 Transgenic Technology

There was some success in the late 1980s and early 1990s in transforming *V. faba* using *Agrobacterium rhizogenes* and *A. tumefaciens* (e.g., Ramsay and Kumar 1990). However, it was not until the beginning of the twenty-first century that it became possible routinely to regenerate transgenic plants of this species (Bottinger et al. 2001). This opens the door for further

development of the crop (see Eapen 2008), such as the introgression of desirable traits from any other organism, including of course the wealth of wild *Vicia* species.

Of the partially domesticated species, genetic transformation has been reported only for *V. narbonensis* and *V. hirsuta*. With the former there has been some success in improving protein quality, mainly by the insertion of a gene encoding a protein from Brazil nut (Pickardt et al. 1995; Muntz et al. 1998). However, in view of the fears expressed about the potential allergenicity of this protein it seems doubtful whether these transgenic narbon beans will be used in human nutrition. An alternative approach has been taken by Rolletschek et al. (2004, 2005, 2007), who have used ectopic gene expression to modify carbon and amino acid metabolism in *V. narbonensis* and have thereby achieved an increase in seed protein content.

For the *V. hirsuta*, the main application has been the production of "hairy roots" after transformation with *A. rhizogenes* to provide material for study of nodulation by symbiotic rhizobia (Quandt et al. 1993).

14.5.5 Genomics, Map-Based Cloning and Marker-Assisted Breeding

Two legumes, namely *Medicago trunculata* and *Lotus japonica*, have been selected as model species for genome sequencing. Annotation of the sequenced genomes is aided by transcriptomics which helps to confirm the identity of genes originally sequenced as expressed sequence tags (ESTs). The establishment of genomic, transcriptomic and proteomic databases (e.g., Cannon et al. 2005; Benedito et al. 2008) also facilitates annotation and thus the identification of "new" genes continues, even after the main sequencing program is "complete" (see, for example, the discussion of plant DNA-polymerase-β: Bryant 2009). However, from the point of view of this chapter, the most important application of the *Medicago* and *Lotus* sequencing projects, together with establishment of the associated databases, is the ability to apply the information to other legumes. One of the reasons for this is the synteny, i.e. the similar way in which genes are ordered along the chromosomes, in different legume species. The comparative mapping across the whole chromosome set has only been performed so far for eight species, amongst which are of course *Medicago* and *Lotus* but also including several crop species (Choi et al. 2004). No *Vicia* species has yet been included in the overall analysis but *Pisum sativum*, another member of the tribe Viceae, is one of the species analyzed. Although inevitably there have been gene rearrangements over millions of years of evolution, the degree of macro synteny between species is remarkable, as are some of the examples of detailed micro-synteny (Choi et al. 2004; see also Cronk et al. 2006). All this provides cause for optimism that *Vicia* species will show similar gene arrangements. This optimism is strengthened by the finding that genetic maps of *V. faba* and *L. culinaris* indicate conservation of genome structure between these two vicioid species (Ellwood et al. 2008).

The next phase in applying this information to breeding of *Vicia* species is the identification of useful markers (see, e.g., van de Ven et al. 1990; Torres et al. 2006; Sanz et al. 2007; Terzopoulos and Bebeli 2008; Varshney et al. 2009). This will aid both marker-assisted selection of useful traits for breeding programs and map-based cloning in order to obtain useful genes for introgression via transgenic technology. The information may also be of value when imported into targeted mutational approaches to the development of new allelic variation via TILLING procedures (Henikoff et al. 2004).

However, given the relatively low agricultural status attributed by many farmers to *Vicia* grains (notwithstanding the enthusiasts already mentioned), it seems likely that breeding progress will, as already hinted, be dependent on the sharing of information and technology within a broader interest in the pulses in general.

14.6 The Dark Side

14.6.1 Introduction

Mention has been made several times of toxic compounds – antinutritional factors (ANFs) – that occur in *Vicia* and indeed in many other legumes. Wagstaff's comprehensive book, *International Poisonous Plants*

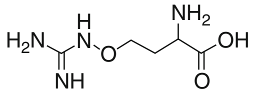
Checklist: An Evidence-Based Reference (2008) lists many *Vicia* species, including most of those that have been used in human and/or animal nutrition over the last ten millennia, as containing toxic compounds. The book contains extensive references to case studies of poisoning episodes. Thus, at the milder end of the scale we learn that a 3-year-old who had ingested seeds of *V. tetrasperma* "suffered only minor gastrointestinal effects which lasted a few hours and had no residual effects upon 24- and 96-h follow-ups" (Wine and Johnson 1993). The toxic compounds in this case were probably lectins and/or protease inhibitors (Oke 2007; Devi et al. 2009; see Sect. 14.6.5).

14.6.2 Nonprotein Amino Acids

The vicioid tribe is part of a group of subfamilies within the Leguminosae which possess the ability to synthesize the non-protein amino acid, canavanine (named after *Canavalia ensiformis*, the plant in which it was first discovered). The distribution of canavanine maps very well onto the cladogram generated by comparison of sequences of the chloroplast *mat*K gene (Wojciechowski et al. 2004) and suggests that canavanine synthesizing ability has evolved only once during the evolution of the legumes.[4]

Canavanine (Fig. 14.8) is an analog of arginine and disrupts several aspects of arginine metabolism (Rosenthal 1977). It is also recognized by arginyl-tRNA synthetase, leading to the incorporation of canavanine into protein; protein function is severely compromized and the aberrant proteins are degraded more rapidly than normal proteins. In plants that synthesize canavanine the arginyl-tRNA synthetase discriminates against canavanine (Bray 1976) thus avoiding the toxic effects on protein synthesis. It is presumed that this ability to "tell the difference" between canavanine and arginine is shown by all the enzymes involved in arginine metabolism in canavanine-synthesizing species.

Fig. 14.8 Canavanine



14.6.3 Cyano-Alanine and Cyanogenic Compounds

γ-L-Glutamyl-β-cyano-L-alanine and β-cyano-L-alanine are synthesized by several *Vicia* species, including *V. sativa*, (Ressler 1962) which is finding increasing use as animal feed. These compounds are neurotoxins,[5] the presence of which was first recognized in the closely related genus, *Lathyrus*. They cause a condition known as lathyrism, which is a painful spastic paralysis of the lower limbs. Over 40 years ago, Ressler et al. (1969) detected toxic amounts of the cyano-alanines in 14 accessions of *V. sativa* and in 30% of the 13 other *Vicia* species tested. Since then, selection in *V. sativa* has led to significant reductions in the levels of these toxins, partly correlated with an increase in seed size (Tate and Enneking 2007b) but no "zero lines" have yet been developed.

The presence of the cyano-alanines in *V. sativa* has also been the subject of a scandalous episode in the commercial use of this species. In the 1980s, Australian breeders produced a red-seeded variety, Blanche Fleur, the seeds of which resemble closely those of red lentil (*L.culinaris*), consumed widely in the Indian sub-continent as red *dahl*. In the late 1980s and onwards into the 1990s, there were several instances of the export from Australia of split seeds of *V. sativa* which were relabeled by the importers and then sold, at a higher price, as a split red lentil (red *dahl*) for human consumption (Tate and Enneking 1992; Tate et al. 1999; Pandian et al. 2002). This led to outbreaks of illness amongst people consuming the seeds and eventually the Australian government dealt with the problem by banning the export of *V. sativa*. Nevertheless, some clandestine export continued for several years after the ban (Tate et al. 1999).

[4]Some taxa have subsequently lost the ability to synthesize canavanine, either naturally during evolution or as a result of selection by humans

[5]Cyano-alanine is, chemically, a non-protein amino acid but does not exert its effects by being incorporated into protein (in contrast to canavanine)

Cyanogenic compounds release the respiratory poison cyanide. Most cyanogenic compounds are glycosides in which the CN moiety is esterified to a sugar molecule; in *Vicia* species the sugar is usually a disaccharide (Poulton 1990). The cyanide is released as HCN in a straightforward hydrolysis. Amongst the more familiar *Vicia* species, *V. angustifolia* is well-known for the presence of vicianin (Poulton 1990); this has also been detected in some accessions of *V. sativa* (Vetter 2000; Ressler and Tatake 2001) and in several other *Vicia* species scattered through the genus (Vetter 2000). Thus some species, exemplified especially by *V. angustifolia* and to a lesser extent by *V. sativa*, synthesize both cyano-alanines and a cyanogenic glycoside.

14.6.4 Favism Inducing Compounds, Vicine and Convicine

Favism is a form of haemolytic anemia (breakdown of red blood cells) caused by ingestion of vicine and convicine. Both these compounds are glycosides of substituted pyrimidines and have a major effect on redox metabolism by rapidly oxidizing reduced glutathione (Jamalian et al. 1976). Vicine also directly inhibits glucose-6-phosphate dehydrogenase (Jamalian et al. 1976) and indeed, favism occurs mainly in people with a genetic deficiency in this enzyme. The term *favism* is derived from *V. faba* but the causative agents, vicine and convicine, are actually widely distributed through the genus.

14.6.5 Lectins and Inhibitors of Proteases

Although their modes of action are very different, these ANFs are generally discussed together (see, for example, Lajolo and Genovese 2002; Oke 2007) because both are proteins. As such, they are denatured and inactivated by heating and thus constitute much less of a problem if the seeds are cooked. Both classes are widely distributed in legumes and the genus *Vicia* is no exception.

Lectins were originally named "phyto-haemagglutinins" because of their ability to agglutinate blood cells. This effect is based on the carbohydrate-binding activity of the lectins. Binding activity is often specific for particular carbohydrate moieties, including those in glyco-proteins found on the surface of blood cells and which are involved in specifying blood group. This type of action may have very serious consequences but in reality, access of the lectins to the circulatory system is usually very restricted if the lectins originate from ingested seeds (Lajolo and Genovese 2002; Oke 2007). Effects of lectins, for example gastro-enteritis, ranging from mild to very severe, are generally ascribed to the ability of lectins to bind to carbohydrate residues in the cell membrane components of the cells lining the alimentary tract (Oke 2007).

Inhibitors of the digestive proteases, trypsin and chymotrypsin, hinder significantly the breakdown of dietary protein and thus reduce the availability of amino acids. In severe cases this leads in turn to hypertrophy of the pancreas. The inhibitors fall into two broad classes, the Kunitz type that inhibit only trypsin and the Bowman–Birk type that inhibit both trypsin and chymotrypsin (see Norton et al. 1985; Lajolo and Genovese 2002; Oke 2007). Both types are small proteins with molecular weights of the order of 23 kDa; the Bowman–Birk type inhibitors are the more stable of the two classes (and therefore more difficult to denature/inactivate) because of a tertiary structure that incorporates many disulphide bridges.

14.6.6 Antinutritional Factors: An Overview

In Sects. 14.6.2–14.6.5, we have discussed the major ANFs that occur in *Vicia* species and in many other legumes. These are not the only toxic compounds that occur in this large genus. Some, such as condensed tannins and other phenolic compounds (Aletor et al. 1994; Berger et al. 2001, 2003) may be less problematic than those discussed above, while others may be confined to only a few species.

Overall therefore, the idea that wider use may be made of the wealth of wild *Vicia* species may seem over-optimistic because of the very widespread occurrence of toxins. Indeed, many species have a veritable

"armory" of chemical weapons, which have presumably evolved as defenses against herbivory. Even in species which have been subject to selection in breeding programs, ANFs are still a problem, as shown by *V. sativa* and by the most extensively selected member of the whole genus, *V. faba*. Despite this, scientists who wish to see much greater use of this genus present a more optimistic view, pointing out the rapidity with which zero-erucic-acid strains of oil-seed rape (canola) were developed. It is suggested that similar concerted efforts with particular *Vicia* species may be similarly rewarded (Tate and Enneking 2007b)

14.7 Concluding Remarks

Vicia is a large and important genus in a large and important family. Land-based biological N-fixation amounts to ca.150 million tons per year, of which the symbiosis between legumes and rhizobia accounts for about one quarter. This has a global environmental significance and is also important in relation to human use of the land. Further, it is definitely realistic to suggest that more *Vicia* species may be domesticated for use as forage and fodder and in human nutrition, provided of course that the problem of ANFs can be solved. However, at most this will involve only a fraction of species in the genus. Nevertheless, as a genetic resource, with the possibility of introgression of desirable traits from wild into domesticated species, the genus *Vicia* is wealthy indeed.

Acknowledgements Anyone who is interested in *Vicia* will soon encounter the extensive work of Dr. Nigel Maxted. His contribution to our understanding the biology of this genus has been immense and it is a pleasure to acknowledge this both on our own behalf and on behalf of anyone with a scientific interest in "the vetches."

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Chapter 15
Vigna

Norihiko Tomooka, Akito Kaga, Takehisa Isemura, and Duncan Vaughan

15.1 Introduction

The economically important *Vigna* species are grown in warm temperate and tropical regions globally but are particularly crucial to human nutrition in large parts of tropical Africa and Asia. *Vigna* also furnish important forage crops. Of the domesticated *Vigna* species, cowpea and mungbean are the most important in terms of production. Cowpea production stands at about 4.5 million metric tons (mmt) of dry grain (seeds) produced on 10 million ha worldwide (*http://www.faostat.fao.org/faostat*). Mungbean is showing an annual increase of about 2.5% in production rates because, as a short duration crop, it fits well into crop rotation cycles. Estimated global production of mungbean is 2.5–3 mt from about 5 million ha (Tomooka et al. 2005). Other *Vigna* species have lower production but are significant contributors to nutrition of some countries or localities for example azuki bean production in Japan is 70–90,000 mt and in China is estimated to be about 700,000 mt (Vaughan et al. 2005). Rice bean (*V. umbellata*) is locally important in parts of northern India and Southeast Asia. Moth bean (*V. aconitifolia*) is an important crop in drier parts of South Asia. Bambara groundnut (*V. subterreanea*) is particularly important in drier parts of Africa.

Norman Borlaug (1973) called the Asian *Vigna* "slow runners" in terms of crop improvement in part because they did not have the benefit of an international (CGIAR) institute to focus research on them. However, the situation for the Asian *Vigna* has recently started to show some significant scientific advances, particularly in relation to genetic resource analysis and genome mapping (Kaga et al. 2005, 2008; Tomooka et al. 2006a).

On the other hand, the main African *Vigna*, cowpea, has been a mandated crop of IITA and consequently has received considerable attention from the international agricultural research community. Despite considerable research attention, seed yield of cowpea remains low in farmer's fields (Singh 2005; Matsunaga et al. 2008). To address this and bring cowpea research and extension on par with other major crops, there have been several research initiatives related to this crop such as the Cowpea Genomics Initiative (Chen et al. 2007), Bean/Cowpea Collaborative Research Support Program (*http://www.isp.msu.edu/CRSP*), and Generation Challenge Program (*http://www.generationcp.org*).

Vigna crops can be viewed in terms of trying to catch up with other major crops. However, in terms of understanding the wild relatives and genetics resources of *Vigna*, the focus of this chapter, the situation is rather better because of the recent publication of two comprehensive books on the Asian and African *Vigna* genetic resources (Tomooka et al. 2002; Maxted et al. 2004). In this chapter, we focus on the scientific progress that has been made in relation to the wild relatives of the cultivated African and Asian *Vigna*.

15.2 *Vigna* in Context

Vigna belong to the hot weather herbaceous legumes, those legumes that do not grow or survive in the winter season in temperate regions (Fig. 15.1). *Vigna* is closely related phylogenetically to the agriculturally important genera *Cajanus*, *Glycine*, and *Phaseolus*.

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C. Kole (ed.), *Wild Crop Relatives: Genomic and Breeding Resources*, *Legume Crops and Forages*,
DOI 10.1007/978-3-642-14387-8_15,

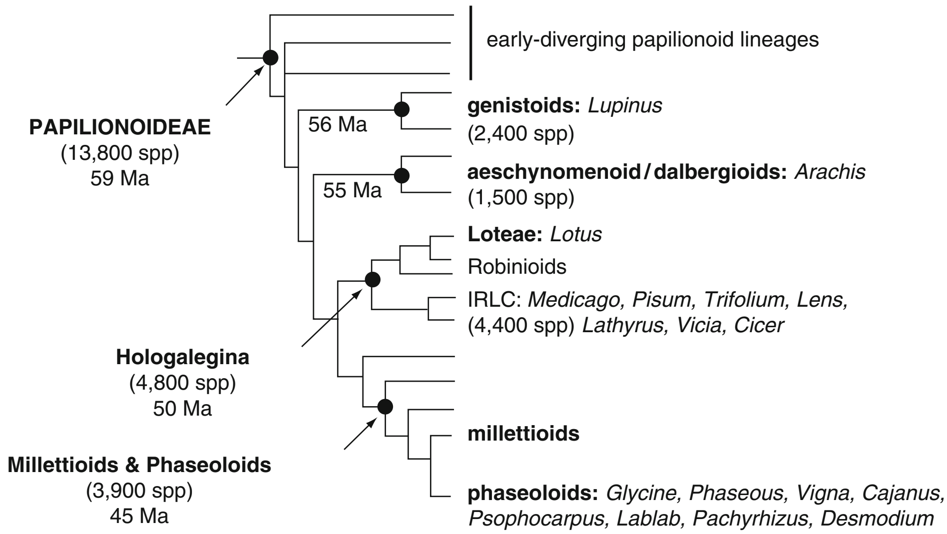


Fig. 15.1 Relationships among papilionoid legume crops [adapted from Gepts et al. (2005)]

Indeed, until 1970, Asian *Vigna* were classified as *Phaseolus* (Verdcourt 1970). The genus *Vigna* consists of over 90 species in six subgenera (Fig. 15.2).

It seems likely that *Vigna* first evolved in Africa because major species radiation of the genus *Vigna* has occurred in Africa where the subgenera *Haydonia*, *Plectotropis*, and *Vigna* are found (Vaillancourt et al. 1993). In addition, molecular studies suggest that *Vigna* may have evolved from the African genus *Wajira* as it is basal compared to *Vigna* and *Phaseolus* (Thulin et al. 2004).

Chloroplast and nuclear genes have enabled molecular clock divergence dates to be estimated for legumes including *Phaseolus* and *Vigna* (Lavin et al. 2004, 2005; Thulin et al. 2004). An estimated date for the divergence between *Vigna subterranean* (subgenus *Vigna*) and *Phaseolus vulgaris* is about 8 million years ago (Mya). Divergence of New World *Vigna* and *Phaseolus* was estimated at about 6 Mya (Delgado-Salinas et al. 2006). The date of divergence of the Asian *Vigna* (subgenus *Ceratotropis*) from African *Vigna* (subgenus *Vigna*) is about 3 Mya and divergence among sections of the Asian *Vigna* about 1 Mya.

Chloroplast DNA (Lavin et al. 2004, 2005; Thulin et al. 2004) and nuclear DNA studies (Lavin et al. 2004) have compared many species of the entire genus *Vigna* and related genera; these studies have provided a new interpretation of the relationships in the genus *Vigna* compared to studies based mainly on morphological traits (Maréchal et al. 1978). Molecular studies suggest that *Vigna* subgenus *Haydonia* of Africa may be the most primitive and well-diverged group within *Vigna*. *Vigna* subgenus *Sigmoidotropis* of the New World is more closely related to *Phaseolus* than to the other subgenera of the genus *Vigna* (Vaillancourt et al. 1993). Among other subgenera,

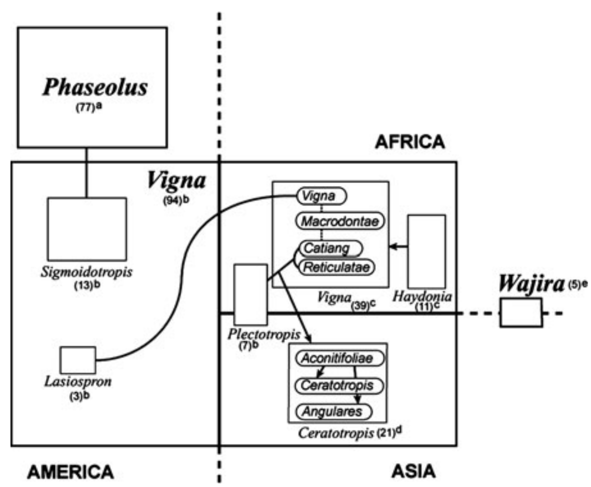


Fig. 15.2 The relationships, geographical distribution, and approximate number of species in the six subgenera in the genus *Vigna* and closely related genera *Phaseolus* and *Wajira*. Approximate species number in each genus and subgenus is shown. Sections within subgenus *Vigna* and subgenus *Ceratotropis* are shown. Modified from Tomooka et al. (2008). Species numbers are from (**a**) Freytag and Debouck (2002), (**b**) Maréchal et al. (1978), (**c**) Maxted et al. (2004), (**d**) Tomooka et al. (2002), and (**e**) Thulin et al. (2004)

subgenus *Vigna* contains a diverse group of species. Species in section *Vigna* subgenus *Vigna* are more closely related to the New World subgenus *Lasiospron*, whereas species in section *Catiang* (containing cowpea) and *Reticulatae* are close to species in the subgenus *Plectotropis*. In Asia, the subgenus *Ceratotropis* represents a homogeneous and distinct group.

The ecological amplitude of the genus *Vigna* is reflected in its global distribution and latitudinal spread (40°N to 35°S). Some species are remarkably salt-tolerant, such as *V. marina*. Others are pyrophytic species, flowering directly from rootstocks following burning, such as *V. ambaciensis* and *V. juncea*. Species in the genus grow from sea level to 3,500 m (Tomooka et al. 2002; Maxted et al. 2004).

15.3 *Vigna* Taxonomy

Maxted et al. (2004) have provided a detailed description of the historical aspects of *Vigna* taxonomy. To date, the most comprehensive taxonomic study of the entire genus was conducted by Verdcourt (1970), which was subsequently modified by Maréchal et al. (1978). They recognized seven subgenera in the genus *Vigna*, namely *Vigna*, *Haydonia*, *Lasiospron*, *Macrorhyncha*, *Plectotropis*, *Sigmoidotropis*, and *Vigna*. Recently, the taxonomy of the subgenus *Macrorhyncha* has been studied, and this subgenus is now recognized as a new genus *Wajira* (Thulin et al. 2004; Fig. 15.2).

According to Maxted et al. (2004), there are 98 species in the genus *Vigna*. Among *Vigna* subgenera, the subgenus *Vigna* has the most species (38) and is divided into six sections. There are about 22 species in the three sections of the Asian *Vigna* (Fig. 15.2).

The main characteristics that distinguish *Vigna* from the very closely related genus *Phaseolus* are the stipule, tubercle (with extra floral nectaries), keel, style, and style beak (Fig. 15.3; Maréchal et al. 1978). The stipule in *Phaseolus* is attached to the stem by its basal part, but it is attached in several ways in *Vigna* (Fig. 15.3a). Tuberacles are present in *Vigna* but not seen in *Phaseolus* (Fig. 15.3b). The keel in *Phaseolus* is narrowly curved around the style, whereas it is variously shaped in *Vigna* as shown (Fig. 15.3c). The style in *Phaseolus* is always curled through 360°, but it may not be curled or curled through 360° in *Vigna* (Fig. 15.3d). In *Phaseolus*, the stigma is at the end of the style, whereas in *Vigna*, the tip of the style sometimes elongates into a style beak so that the stigma is situated laterally to the style (Fig. 15.3e).

15.4 Conservation

15.4.1 *Ex situ conservation*

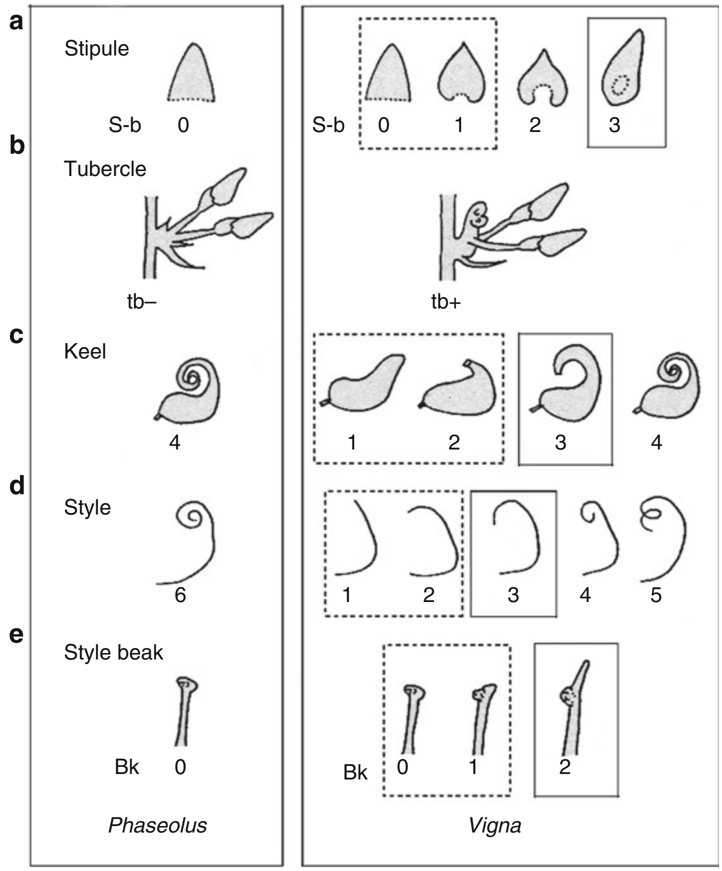
15.4.1.1 Asian *Vigna*

There are now accessions of all known Asian *Vigna* species in ex situ germplasm collections. This reflects the concentrated collection efforts focusing on wild species by collectors, particularly those led by N. Tomooka from Japan and R.J. Lawn from Australia. Since the review of Asian *Vigna* conservation status (Tomooka et al. 2002), specimens of *V. dalzelliana* and *V. khandalensis* have been collected and are conserved at the Tamil Nadu Agricultural University, India.

Recent extensive collection of Asian *Vigna* has revealed the true status of Asian *Vigna* in several countries (Fig. 15.4a–f). It is surprising that even in countries with rather well-studied flora, such as Sri Lanka, many new Asian *Vigna* have been recorded for the first time. The Revised Handbook of the Flora of Ceylon recognizes only two wild Asian *Vigna,* when in fact six are present. Germplasm collection reports in English that have focused on Asian *Vigna* collections can be found at website: (*http://www.gene.affrc.go.jp/publications.php?type=report&section=plant*).

A comprehensive molecular analysis of wild *Vigna* germplasm from Thailand that is a center of diversity for wild Asian *Vigna* has been conducted (Seehalak et al. 2006). The study revealed that rice bean (*V. umbellata*) probably was domesticated there or in a neighboring region (Seehalak et al. 2006). In addition, wild *V. mungo* from Thailand is distinct from Indian wild black gram. Intraspecific genetic diversity was high for several species, and the species *V. hirtella* appears to have evolved two distinct forms. The high level of wild *Vigna* diversity in northern Thailand, where as many as five species can be found at one site, suggests that this region is well-suited to development of in situ conservation reserves.

Fig. 15.3 Key traits that distinguish *Phaseolus* and *Vigna* [modified from Tomooka et al. (2002)]. *Boxes* surround traits of subgenus *Ceratotropis*, and *dashed boxes* surround traits of subgenus *Vigna* section *Catieng* (cowpea group)



15.4.1.2 African *Vigna*

Maxted et al. (2004) reviewed the status of conservation of African *Vigna* in detail. About 10,000 accessions of cowpea and 1,500 accessions of bambara ground nut are conserved ex situ. However, the review highlighted the fact that little attention that has been paid to the conservation of the wild species. More than 20 species of African *Vigna* are apparently not conserved in any ex situ collection even though several of these species have ethnobotanical uses (Table 15.1).

15.4.2 Core Collections

Germplasm in genebanks is there for conservation and use. To enable the increasing numbers of conserved accessions to be rationally and cost effectively evaluated for use, a core collection approach has been widely applied (Hodgkin et al. 1995). A core collection aims to represent about 90% of the allelic diversity in 10% of the accessions, while a mini-core collection aims to represent about 80% of the allelic diversity in 1% of the accessions (Upadhyaya and Ortiz 2001). Other approaches have been developed to make germplasm more usefully available for study such as a representative Asian *Vigna* species collection that uses one or two accessions of each taxon to understand the diversity for different traits across the subgenus (Konarev et al. 2002; Tomooka et al. 2000, 2002).

Core collections have been developed for the major *Vigna* crops – cowpea (Mahalakshmi et al. 2007), mungbean (Bisht et al. 1998; Sangiri et al. 2007), and azuki bean (Xu et al. 2008) (Table 15.2). Some of them have incorporated wild germplasm into these core collections (e.g., Sangiri et al. 2007). Study of

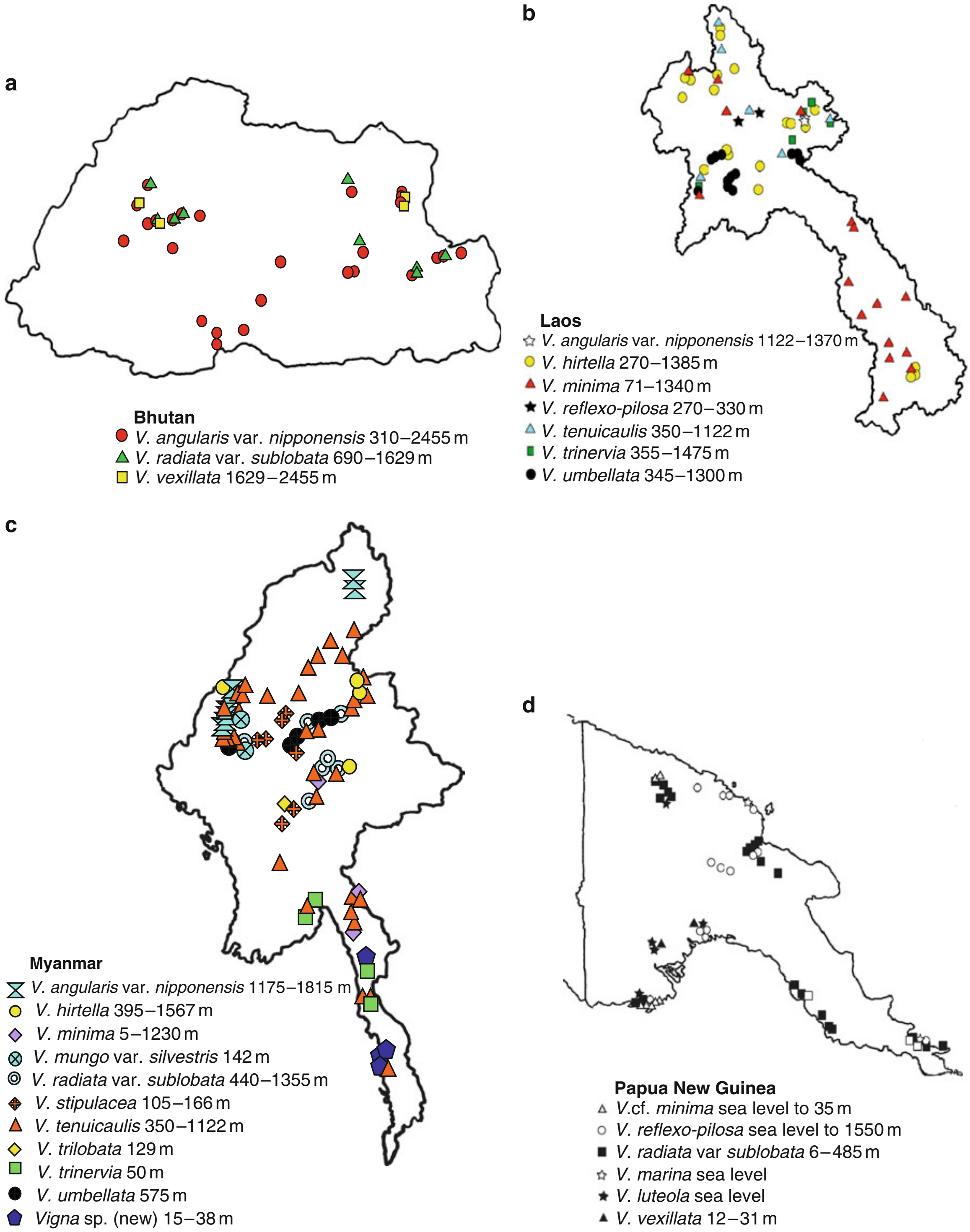


Fig. 15.4 (continued)

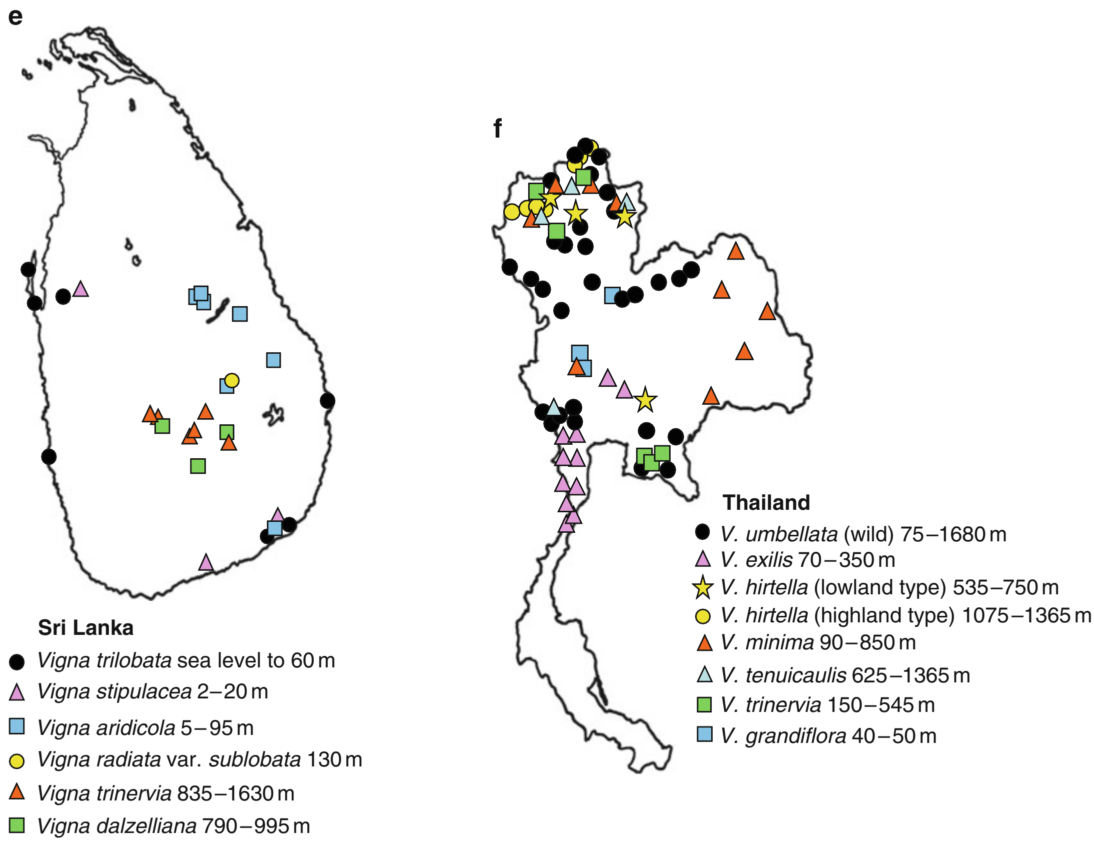


Fig. 15.4 Distribution of wild *Vigna* species in countries that have recently been surveyed. (**a**) Bhutan, (**b**) Laos, (**c**) Myanmar, (**d**) Papua New Guinea, (**e**) Sri Lanka, (**f**) Thailand

crop complex variation has enabled the breadth of crop diversity to be incorporated into core collections. Such genetic diversity studies have also revealed previously unknown or poorly known regions of genetic diversity. For example, analysis of azuki bean variation showed that the Himalayan region wild germplasm has much greater genetic variation than other regions (Zong et al. 2003). Simple sequence repeat (SSR) analysis revealed that Nepalese azuki germplasm consisted of two groups of materials: one group from eastern Nepal was similar to Bhutanese azuki germplasm, while in western Nepal, azuki germplasm was more similar to Chinese azuki germplasm (Xu et al. 2008). Analysis of diverse mungbean germplasm revealed Australia and New Guinea wild mungbean as a distinct center of diversity for wild mungbean (Sangiri et al. 2007).

Another approach to developing useful sets of germplasm for evaluation has been the representative species collection (Tomooka et al. 2000). This approach has enabled species level evaluation to quickly highlight useful germplasm. Using an Asian *Vigna* species collection, many new sources of resistance to bruchid beetles have been found (Tomooka et al. 2000), and this approach has enabled more detailed studies of the most promising resistance sources (Kashiwaba et al. 2003). The Asian *Vigna* species representative collection was evaluated for proteinase inhibitor variation and species with very low levels or absence of trypsin inhibitors were identified (Konarev et al. 2002).

Table 15.1 Ethnobotany of *Vigna* species (cultivated or domesticated taxa in bold, common name in parenthesis)

Species	Subgenus, section	Natural distribution	Uses
***V. aconitifolia* (Moth bean)**	Ceratotropis, Aconitifoliae	South Asia	Pulse, green pods as vegetable, forage, cover crop, green manure (Maxted et al. 2004)
V. adenantha	Sigmoidotropis, Laptospron	Americas	Medicinal uses, tuberous roots eaten (Maxted et al. 2004)
V. adenantha	Sigmoidotropis, Laptospron	Americas	Tubers of wild type eaten (Maxted et al. 2004)
V. ambacensis	Vigna, Vigna	Africa	Tubers eaten by human, leaves grazed by animal and used as fodder, leaves dried and smoked as a cough remedy (Maxted et al. 2004)
V. angivensis	Vigna, Vigna	Madagascar (endemic)	Tuberous roots, pods and seeds eaten by domesticated and wild animals (Maxted et al. 2004)
***V. angularis* (Azuki bean)**	Ceratotropis, Angulares	Himalaya to East Asia	Pulse, sweet soup or paste, celebration (Tomooka et al. 2002), green pods as vegetable [Nepal, (Personal communication from Dr. Jwala Baajracharya, Nepal Agric. Res. Council)]
V. angularis	Ceratotropis, Angulares	Himalaya to East Asia	Wild and weedy type collected and eaten in times of food shortage (Yamaguchi 1992)
V. antillana Pulse, soil improvement (Cuba) (Maxted et al. 2004)	Sigmoidotropis,	Sigmoidotropis	Caribbean
***V. caracalla* (Snail flower)**	Sigmoidotropis, Caracallae	South and Central America	Ornamental, minor pulse (Cuba) (Maxted et al. 2004)
V. fischeri	Vigna, Vigna	East Africa	Flower and roots eaten by humans, the raw root has a sweet juice, tubers eaten by human, leaves and shoot used to make rope (Maxted et al. 2004)
V. friesiorm	Vigna, Macrodontae	East Africa	All plant parts including tubers eaten by baboons (Maxted et al. 2004)
V. frutescens	Vigna, Liebrechtsia	East Africa	All plant parts including tubers eaten by baboons and grazing animals, stem used to make rope, flower fragrant may have potential as a ornamental (Maxted et al. 2004)
V. gracilis	Vigna, Vigna	East Africa	Seeds and pods eaten by humans after grilled, roots used as a vermifuge, upper plant parts grazed by animals and used as fodder (Maxted et al. 2004)
***V. hosei* (Sarawak bean)**	Vigna, Vigna	East Africa, South and Southeast Asia	Cover crop, green manure (India, Malaysia, Sri Lanka) (Maxted et al. 2004)
V. juncea	Haydonia, Haydonia	Central Africa	Fruit and seed eaten by humans (Maxted et al. 2004)
***V. luteola* (Dalrymple vigna)**	Vigna, Vigna	Africa (now prevailed pan-tropical area)	Fodder and forage (Argentina), soil improvement (Cuba) (Maxted et al. 2004)
V. luteola (wild plants)	Vigna, Vigna	Africa (now prevailed pan-tropical area)	Upper plant parts grazed by animals (Maxted et al. 2004)
V. macrorhyncha	Macrorhyncha	Africa	Roots eaten by humans as raw roots (Maxted et al. 2004)
***V. marina* (Dune bean, Beach bean)**	Vigna, Vigna	Africa (now prevailed pan-tropical area)	Pulse for human [Indian Ocean (Tomooka et al. 2004)], cover crop (Southeast Asia), forage (Cuba) (Maxted et al. 2004)
V. marina (wild plants)	Vigna, Vigna	Africa (now prevailed pan-tropical area)	Shoots grazed by animals and used as fodder. Seeds used as coffee substitute (Gabon). Tubers eaten by humans (Maxted et al. 2004)
V. membranacea	Vigna, Macrodontae	East Africa	Flower, fruit, seed and root eaten by humans (Maxted et al. 2004)
V. minima	Ceratotropis, Angulares	Southeast and East Asia, Papua New Guinea	Flowers and young pods eaten by humans (Laos) (Tomooka et al. 2004)

(*continued*)

Table 15.1 (continued)

Species	Subgenus, section	Natural distribution	Uses
V. monophylla	Haydonia, Haydonia	East Africa	Roots eaten by humans (Maxted et al. 2004)
***V. mungo* (Black gram, urd bean)**	Ceratotropis, Ceratotropis	South Asia	Pulse (South Asia), green pods as vegetable, forage, green manure (Maxted et al. 2004), bean sprout [Japan (Tomooka et al. 2002)]
V. nuda	Plectotropis,	Pseudoliebrechtsia	Central Africa
Flavoring in beer (Maxted et al. 2004)			
V. oblongifolia	Vigna, Vigna	Tropical Africa	Roots eaten by humans as raw roots (Maxted et al. 2004). Flower structure similar to *V. luteola* (Maréchal et al. 1978).
***V. parkeri* (Creeping vigna)**	Vigna, Vigna	Central and East Africa	Pasture (Australia and USA), cover and green manure (New Guinea). Cover crop under coconuts (Madagascar) (Maxted et al. 2004)
V. parkeri (wild plants)	Vigna, Vigna	Central and East Africa	Above ground parts grazed by animals as forage (Maxted et al. 2004)
***V. radiata* (Mungbean, green gram)**	Ceratotropis, Ceratotropis	East Africa through tropical Asia to Papua New Guinea, tropical Australia	Pulse (South Asia), noodles (vermicelli), bean sprout and sweet soup or paste (Southeast and East Asia) (Maxted et al. 2004; Tomooka et al. 2002)
***V. radiata* var. *sublobata* (wild plants)**	Ceratotropis, Ceratotropis	East Africa through tropical Asia to Papua New Guinea, tropical Australia	Boiled seeds eaten by humans (India) (Janardhanan et al. 2003)
V. racemosa	Vigna, Vigna	Tropical Africa	Leaves and shoots eaten by chimpanzees and other animals. Mashed-up leaf drunk for cataracts and catarrh (Maxted et al. 2004). Leaf used as poultice for testicles (Maxted et al. 2004)
***V. reflexo-pilosa* var. *glabra* (=*V. glabrescens*)(Creole bean)**	Ceratotropis Angulares	Southeast Asia, Oceania	Pulse (West Bengal, Mauritius, Vietnam, Philippines) (Tomooka et al. 2002)
V. reticulata	Vigna, Reticulatae	Tropical Africa	Tuberous roots eaten by humans (Maréchal et al. 1978) Leaves eaten by humans. Leaves and shoots eaten by animals as forage. Sap decoction used for ear-ache and diarrhea (Tanzania) (Maxted et al. 2004)
V. stenophylla	Vigna, Vigna	East Africa	Tuberous roots eaten by humans (Maxted et al. 2004)
***V. stipulacea*[a]**	Ceratotropis, Aconitifoliae	South Asia	Pulse and green manure (India) (Tomooka et al. 2008), forage, cover crop (India, Pakistan, Indonesia and Sudan) (Maxted et al. 2004), Seeds are roasted and eaten by humans (India) (Janardhanan et al. 2003)
***V. subterranea* (Bambara groundnut)**	Vigna, Vigna	Sub-Saharan Africa	Pulse, green pods as vegetable (Africa, Madagascar) (Maxted et al. 2004)
V. subterranea	Vigna, Vigna	Sub-Saharan Africa	Fruit and seed eaten raw by humans (Maxted et al. 2004)
***V. trilobata*[a]**	Ceratotropis, Angulares	South Asia and Myanmar	Young pods, seeds are roasted and eaten by humans (India) (Tomooka personal observation 2009)
***V. trinervia* (Tua pee)**	Ceratotropis, Angulares	South and Southeast Asia	Cover crop to suppress weed growth in rubber plantation (Thailand) (Tomooka et al. 2002)
***V. umbellata* (rice bean)**	Ceratotropis, Angulares	South and Southeast Asia	Pulse, green pods as vegetable, fodder, green manure (Maxted et al. 2004; Tomooka et al. 2002)

(*continued*)

Table 15.1 (continued)

Species	Subgenus, section	Natural distribution	Uses
V. umbellata (wild plants)	Ceratotropis, Angulares	South and Southeast Asia	Flowers and young pods eaten as vegetables (Laos) (Tomooka et al. 2006b). Wild deer like to eat shoots (Myanmar)
***V. unguiculata* (cowpea, yard long bean)**	Vigna, Catiang	Africa (cultivated worldwide)	Pulse (cowpea), green pods as vegetable (yard long bean, Asia), sweet soup or paste (cowpea, East and Southeast Asia)
V. unguiculata (wild plants)	Vigna, Catiang	Africa	Leaves, shoots, young pods and seeds eaten by humans. Tuberous roots eaten by humans (may be poisonous when roasted). Roots used as dye (Maxted et al. 2004)
***V. vexillata* (root cowpea)**	Plectotropis, Plectotropis	Pan tropical	Tubers eaten by humans. Domesticated plants grown in Bali and Timor for edible tuber (Karuniawan et al. 2006). Wild plants used for pasture, green manure, cover crop and edible tuber.
V. vexillata (wild plants)	Plectotropis, Plectotropis	Pan tropical (originally Africa)	Leaves, pods and tubers eaten by humans. Tubers may be used as a water source. Leaf decoction is used as a wash against itch. Roots ground into a paste for tropical ulcers and sores (Mozambique) and for schistosomiasis (Tanzania) (Maxted et al. 2004) Seeds used to prepare curry (Nilgiri Hills, Tamil Nadu) (Tomooka et al. 2008) Seeds boiled and eaten (Northeast India) (Janardhanan et al. 2003, treated as *V. capensis*). Seeds are roasted and eaten by Khatkharis and Ghonds (India) (Janardhanan et al. 2003, treated as *V. vexillata*)

[a]We had previously considered that reports of *V. trilobata* being used as a food may have been the result of mistaken identification of *V. stipulacea* (Tomooka et al. 2006a). However, during a recent visit to South India by the senior author, it has been confirmed that both *V. trilobata* and *V. stipulacea* are used as an occasional food there. Variation in *V. trilobata* suggests that it may in some places be semi-domesticated

Table 15.2 Core collection

Crop	No. of accession (wild sp.)	Traits	Core	No. acc	References
Cowpea	10,227 (64)	28 agronomic	Core	2,062 (6)	Mahalakshmi et al. (2007)
Mungbean	1,532	38	Core	152	Bisht et al. (1998)
Mungbean	615 (189)	19 SSR primers	Core	106 (54)	Sangiri et al. (2007)
Azuki bean	616 (67 and 1 weedy)	13 SSR primers	Core	96 (9)	Xu et al. (2008)

15.5 Cross Compatibility

The crop gene pool concept developed by Harlan and de Wet (1971) provides a basis for understanding which wild species related to particular crop can be most readily used by traditional breeding methods (primary gene pool) and which species require special techniques to effect gene transfer into a particular crop (secondary and tertiary gene pools).

15.5.1 *Asian* Vigna

The primary gene pool of azuki bean (*Vigna angularis*) consists of its wild, weedy, and cultivated forms (Fig. 15.5a). The secondary gene pool consists of two parts. Secondary gene pool (a) consists of species that cross relatively easily both as seed parent and as pollen parent with *V. angularis* – *V. minima*, *V. nakashimae*, *V. nepalensis*, *V. riukiuensis*, and *V. tenuicaulis*.

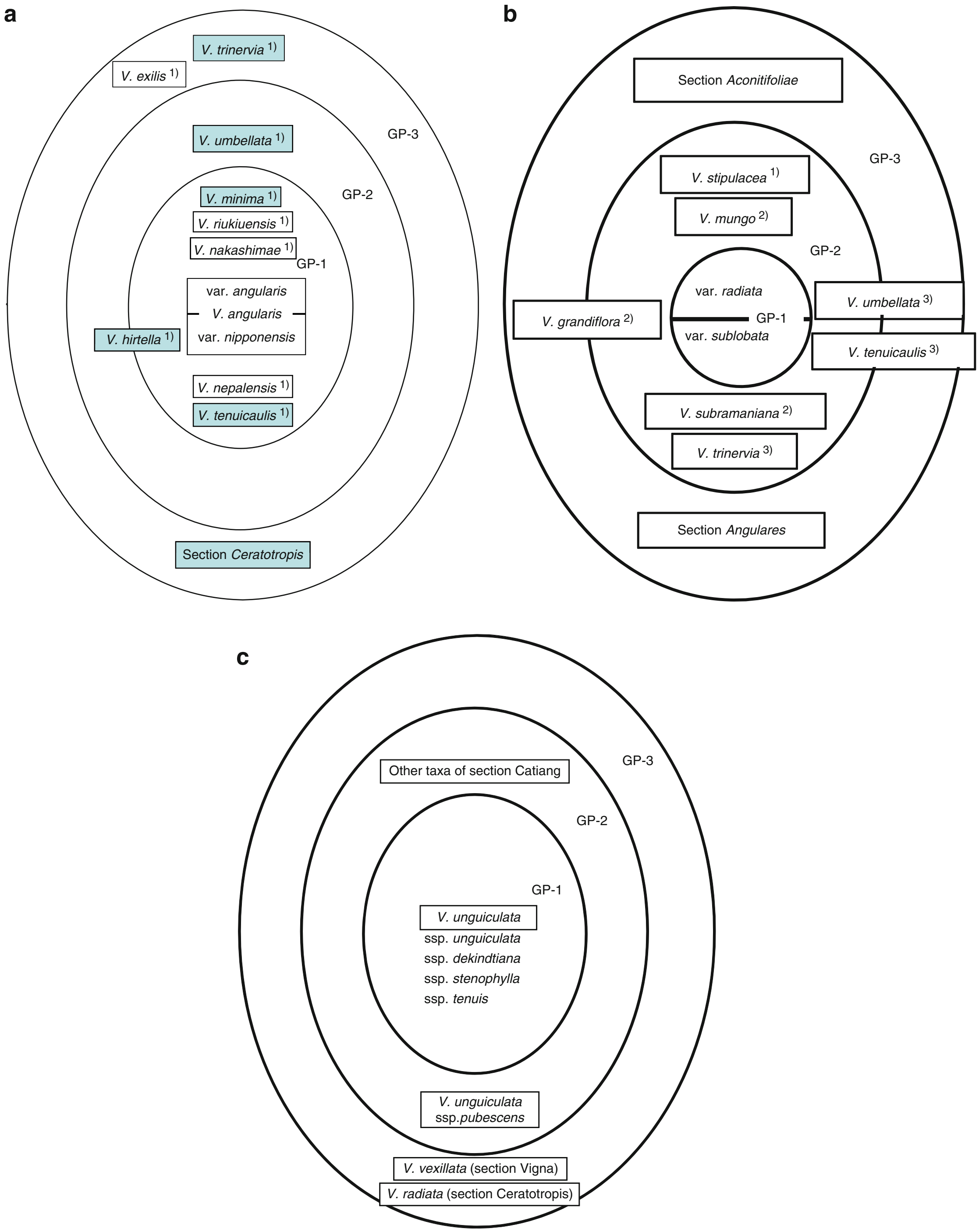


Fig. 15.5 Gene pools of major *Vigna* crops. Gene pool 1 (GP-1) constitutes the biological species. Gene pool 2 (GP-2) includes these species that cross with GP-1 with at least some fertility; Gene pool 3 (GP-3) includes those species where gene transfer requires radical techniques. (**a**) Azuki bean. (1) Species in section Angulares. Some of the species in the section Ceratotropis have not yet been examined for their cross compatibility relationships with azuki bean and therefore this section is tentatively classified as GP-3. (**b**) Mungbean. (1) This species is in section Aconitifolia. (2) These species are in section Ceratotropis. (3) These species are in section Angulares. (**c**) Cowpea [(**a**) and (**b**) updated from Tomooka et al. (2005) and Vaughan et al. (2005)]

V. nakashimae has been successfully used as a bridging species along with other species in the *V. minima* complex (Tomooka et al. 2006a). Secondary gene pool (b) consists of *V. hirtella* that cross either as female (seed) parent or can cross in both directions, depending on the accession. *V. umbellata* is also in the secondary gene pool (b) and can only produce hybrids with azuki bean with the help of embryo rescue. It is probable that other species in section Angulares, such as *Vigna exilis* and *V. dalzelliana,* are part of the secondary gene pool, but these species have yet to be studied. Although there is limited information, the tertiary gene pool would be species in section Ceratotropis (*Vigna radiata*, *V. mungo,* etc.) gene pool. Cross compatibility of species in section Aconitifoliae (*V. aconitifolia*, *V. trilobata,* etc.) with azuki bean has not been clarified (Tomooka et al. 2002).

The gene pools of mungbean consist of a primary gene pool of domesticated *V. radiata* and its presumed wild progenitor var. *sublobata* (Fig. 15.4b). The secondary gene pool of mungbean consists of the other species in section Ceratotropis, *V. mungo*, *V. subramanian*, *V. grandiflora*, as well as *V. stipulacea* (Section Aconitifolia), *V. tenuicaulis*, *V. trinervia*, and *V. umbellata* (Section Angulares). Other species in sections Aconitifolia and Angulares are within the tertiary gene pool based on studies of the first author to date.

Dana and Karmakar (1990) proposed two genome groups for the subgenus *Ceratotropis*, AA and A_1A_1, based on crossability, hybrid fertility, and chromosome pairing. The genome group AA includes mungbean and *Vigna aconitifolia*, *V. dalzelliana*, *V. khandalensis*, *V. mungo*, and *V. trilobata*, while A_1A_1 consists of *V. angularis* and *V. umbellata*. There have been many reports of interspecific hybridization involving mungbean (for reviews, see Dana and Karmakar 1990; Tomooka et al. 2002). Results of interspecific hybridization suggests that mungbean and related Asian *Vigna* have more complexity to genome structure than indicated by just two groups for the 21 species in the subgenus *Ceratotropis*. A complete study and synthesis of information of genomes in the subgenus *Ceratotropis* have yet to be undertaken.

15.5.2 African Vigna

V. vexillata (subgenus *Plectotropis*) has been the focus of efforts to develop hybrids with *V. unguiculata* (subgenus *Vigna*) due to its range of useful traits, particularly cowpea mottle carmovirus. However, these have not been successful (Evans 1976; Barone and Ng 1990). Therefore, alternative approaches to introducing useful traits from this species into cowpea are being sought, such as seeking a bridging species, gene cloning, and transformation of cowpea.

An attempt to develop a bridging cross employed a hybrid between *V. vexillata* and *V. davyi* also of subgenus *Plectotropis*. The F_1 was partially fertile, but when crosses with cowpea were attempted, no hybrid was obtained (Fatokun et al. 1996).

A similar approach with similar results occurred when the partially fertile hybrid between *V. oblongifolia* and *V. luteola* (subgenus *Vigna* Section Vigna) was crossed with cowpea to try and transfer insect resistance. No hybrid was obtained (Fatokun et al. 1996).

Within the complex section *Catiang* of the subgenus *Vigna*, there is some cross incompatibility. To transfer pubescence from *V. unguiculata* ssp. *dekindtiana* var. *pubescens* to cowpea, a hybrid was successfully made, but it entailed embryo rescue (Fatokun et al. 1996). A cross between cowpea and *V. unguiculata* ssp. *rhomboidea* resulted in an F_2 population, but about 30% of plants produced no pods. A cross between a yard-long bean (ssp. *sesquipedalis*) and ssp. *tenuis* resulted in an F_1 with 60% pollen fertility and pods with few seeds. These examples suggest that even within a section of the genus *Vigna* there are various types of incompatibility (Fatokun et al. 1996).

These studies provide a basis for understanding the complexity of the crop gene pool system. For cowpea, studies suggest that the primary gene pool consists of the four cultivar groups of *V. unguiculata* ssp. *unguiculata* and *V. unguiculata* ssp. *dekindtiana*, *stenophylla*, and *tenuis* (Fig. 15.4c). The secondary gene pool consists of some subspecies of *V. unguiculata,* such as ssp. *pubescens*. *V. vexillata* (subgenus *Vigna*), and *V. radiata* (subgenus *Ceratotropis*) represent species in the tertiary gene pool.

15.6 Evaluation

Wild germplasm is not a preferred source of useful genes for breeding because of its use requires backcrossing to get rid of unwanted genes. However, wild *Vigna* are a source of many useful genes not found in the cultigen gene pools.

1. Resistance to bruchids have observed in *V. riukiuensis*, *V. reflexo-pilosa* (Tomooka et al. 1992), *V. radiata* var. *sublobata* from Madagascar (Fujii and Miyazaki 1987; Kaga and Ishimoto 1998) and from Australia (Miyagi et al. 2004), *V. umbellata* (Tomooka et al. 2000; Kashiwaba et al. 2003; Somta et al. 2006), *V. tenuicaulis* (Tomooka et al. 2000), and *V. nepalensis* (Somta et al. 2008a).

   In the African *Vigna, V. vexillata, V. reticulata, V. oblongifolia*, and *V. luteola* were found to have a high level of resistance to cowpea storage weevil (Ng 1990).
2. *V. stipulacea* shows high resistance to powdery mildew due to hypersensitive reaction (Tomooka et al. 2006a). *V. reflexo-pilosa* var. *glabra* has also been reported to have powdery mildew resistance (Egawa et al. 1996).
3. Low trypsin inhibitor activity has been found in *V. tenuicaulis* (Konarev et al. 2002). Chymotrypsin was not detected in *V. grandiflora* (Konarev et al. 2002).
4. High methionine content has been reported in seeds of *V. radiata* var. *sublobata* (Babu et al. 1988).
5. High photosynthetic efficiency and drought tolerance has been reported in *V. raditata* var. *sublobata* (Ignacimuthu and Babu 1987).
6. Moth bean (*V. aconitifolia*) is a good source of drought tolerance (Jain and Mehra 1980).
7. Moth bean (*V. aconitifolia*) has the highest heat tolerance among the 15 Asian *Vigna* species tested. It could survive being subjected to consecutive heat treatment conditions of 36° for 12 days, 38° for 5 days, and 40° for 11 days. All other tested species died in the same test under 40°C temperatures (Tomooka et al. 2001). *V. riukiuensis* is resistant to heat stress (Egawa et al. 1999). Since heat stress is a constraint to growing azuki beans in tropical and subtropical areas and even in northern Japan, attempts have been made to identify and transfer genes for resistance to heat stress in *V. riukiuensis* to azuki bean. Several quantitative trait loci (QTLs) for resistance to heat stress have been identified; among them, two (HQTL1 and 2) maintain pollen viability during extended heat stress. Currently, a backcrossing program is underway to incorporate these QTLs into azuki breeding lines (Kaga et al. 2003).
8. Sources of resistance to the parasitic plant species in the genera *Striga* and *Alectra*, important mainly in Africa, have been found in landraces and breeding lines of cowpea (Singh 2005). So the wild gene pool has not yet been needed for resistance.
9. Parthenocarpy has been found in crosses between wild and cultivated cowpea that may enable apomixes to be identified and lead to the development of hybrid cowpeas (Hall et al. 1997).
10. Insect resistance in the form of increased pubescence from *V. unguiculata* ssp. *dekindtiana* var. *pubescens* has been introduced into cowpea breeding lines (Ehlers and Hall 1997).
11. Pronounced antibiosis to cowpea moth (*Cydia ptychora*) was detected in the wild species *V. unguiculata* ssp. *mensensis* (Ezueh 1981).
12. Yellow mosaic virus resistance has been reported in *V. radiata* var. *sublobata* (Singh and Ahuja 1977). Cucumber mosaic virus resistance has been reported in *V. reflexo-pilosa* var. *glabrescens* (Egawa et al. 1996). Cowpea mottle carmovirus (CPMoV) resistance has been reported in *V. vexillata* (Thouttappilly et al. 1994; Ogundiwin et al. 2002).
13. Bean fly (*Ophiomyza phaseoli*, *O. centrosematis* and *Melanagromyza soja*) resistance has been reported in the cultivated form of the tetraploid species *V. reflexo-pilosa* (Egawa et al. 1996).
14. Flower thrips, pod sucking bugs, and mucuna pod borer has been found in *V. unguiculata* ssp. *dekindtiana* var. *pubescens* and *V. unguiculata* ssp. *tenuis* (Ehlers and Hall 1997) and *V. vexillata* (Ng 1990).
15. High tolerance to saline and alkaline soils has been reported in *V. radiata* var. *sublobata* (Lawn et al. 1988).
16. Resistance to pod bug (*Clavigralla tomentosicollis*) has been identified in the wild cowpea (*V. unguiculata* ssp. *dekinditiana* TVNu 151) (Koona et al. 2002).
17. Many *V. vexillata* lines have been identified as having high levels of resistance to several cowpea insect pests including pod-sucking bug (*C. tomentosicollis*), the bruchid *Callosobruchus maculatus*, and the pod borer *Maruca vitrata* (Birch et al. 1986; IITA 1988).
18. One of the important considerations of growing legume crops is the efficiency with which they fix nitrogen and may increase soil fertility. In a

survey of *Bradyrhizobium* in the root nodules of various wild and cultivated *Vigna* from Thailand, much greater variation was observed in *Bradyrhizobium* from Thailand than in soybean *Bradyrhizobia* from Thailand, Japan, and the US (Yokoyama et al. 2006). This study suggested that useful variation exists in *Bradyrhizobium* to improve symbiotic systems.

19. Resistance to soybean cyst nematode (*Heterodera glycines*) has been found in *V. riukiuensis*, and this trait is being transferred to azuki bean in Japan (Shimada, Tokachi Experiment Station, 2009, personal communication).

15.7 Genome Mapping and Domestication Syndrome

Wild relatives of crops provide insights into crop evolution and the domestication syndrome. The domestication syndrome can help to understand how the crop has evolved and also how the crop may further evolve. Wild species have, therefore, been important in the development of mapping populations that have led to abundant information on the location of QTL of potential for crop improvement. A summary of genome maps published for various *Vigna* species is shown (Table 15.3).

15.7.1 Azuki Bean

Wild azuki beans have a higher yield on per plant basis than domesticated azuki beans. This is because domestication of azuki bean has involved a trade-off between yield and seed size. Domesticated azuki bean has fewer longer pods and fewer larger seeds on plants with shorter stature compared to wild azuki bean (Kaga et al. 2008).

The domestication syndrome in azuki bean has been studied in detail in two different crosses (Isemura et al. 2007a; Kaga et al. 2008). One cross was of azuki bean and a closely related species from the Himalayan region, *V. nepalensis*. The second involved azuki bean and an accession of its wild progenitor from Japan, *V. angularis* var. *nipponensis*. Both studies showed similarities: about 40% commonality in QTLs detected and clusters of domestication-related QTLs clustered on the same linkage groups 1, 4, 7, and 9. However, the two studies revealed some differences. One difference was the detection of a presumed translocation in the cross between azuki bean and wild azuki from Japan (Kaga et al. 2008).

15.7.2 Black Gram

Two genome maps have been developed in black gram (Chaitieng et al. 2006; Gupta et al. 2008). These maps show linkage order of markers is highly conserved between black gram and azuki bean. These maps were developed using simple sequence repeat (SSR) markers from azuki bean as well as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and intersimple sequence repeat (ISSR) markers. However, both maps had significant gaps that may require development of specific molecular markers for black gram.

15.7.3 Cowpea

The development of linkage maps for cowpea has concentrated on identifying the location of QTLs for various agronomically important traits. Genome maps that have used one cultivated and one wild plant have focused on identifying morphoagronomic traits (Fatokun et al. 1992; Ubi et al. 2000) and aphid resistance (Myers et al. 1996). However, the most detailed genetic maps for cowpea have been developed from a cross between two cultivated parents that have complementary useful traits such as resistance to different races of *Striga*, cowpea mosaic virus, root-knot nematode virus, and *Fusarium* wild (Menéndez et al. 1997; Ouédraogo et al. 2001, 2002a, b).

15.7.4 V. vexillata

V. vexillata is a highly variable pan-tropical species that is palatable to cattle; its green pods are also used as fresh vegetable, and its tubers are eaten by some

Table 15.3 *Vigna* genome linkage maps

Cross combination	Population (plants/lines) analyzed	Markers used	LG[a]	Map distance	Level of distortion	References
V. angularis (cv. Erimoshouzu) × *V. nakashimae*	F_2 population (80)	19 RFLP, 108 RAPD and 5 morphological markers	14	1,250 cM	0.197	Kaga et al. (1996)
V. angularis (cv. Erimoshouzu) × *V. umbellata* (cv. Kagoshima)	F_2 population (86)	114 RFLP, 74 RAPD, 1 morphological marker	14	1,702 cM	0.298	Kaga et al. (2000)
V. radiata var. *radiata* × *V. radiata* var. *sublobata* (from Madagascar)	F_2 population (58)	151 RFLP, 20 cDNA and 1 pest locus	14	1,570 cM	0.12	Menacio-Hautea et al. (1992)
V. radiata var. *radiata* × *V. radiata* var. *sublobtata* (from Australia)	F_2 population (67)	52 RFLP, 56 RAPD, 2 morphological markers	12	758.3 cM	0.145	Lambrides et al. (2000)
V. radiata var. *radiata* × V. *radiata* var. *sublobata* (from Australia)	Recombinant inbreed (67)	113 RAPD, 2 morphological markers	12	691.7 cM	0.24	Lambrides et al. (2000)
V. radiata var. *radiata* × *V. radiata* var. *sublobata* (from Australia)	Recombinant inbreed (227)	78 RFLP markers (64 probes) 1 morphological locus	13	684.7 cM	0.367	Humphrey et al. (2005)
V. radiata (cv. Berken) × *V. radiata* ssp. *sublobata* (ACC41)	Recombinant inbreed (80)	255 RFLP markers	13	737.9 cM	0.308	Humphrey et al. (2002)
V. unguiculata (IT84S-2246-4 improved line) × *V. unguiculata* ssp. *dekindtiana* var. *pubescens* (TVNu-110-3A)	Recombinant inbreed (94)	77 RAPD, 3 morphological markers	12	669.8 cM	0.217	Ubi et al. (2000)
V. unguiculata (IT2246-4) × *V. unguiculata* ssp. *dekindtiana* (TVN1963)	F_2 population (58)	79 genomic4 cDNA, 6 RAPD, 2 aphid, 1 seed coat texture markers	10	>800 cM	0.22	Menacio-Hautea et al. (1993) and Fatokun et al. (1997)
V. unguiculata (IT84S-2049) × *V. unguiculata* (524B)	F_2 population (94)	133 RAPD, 19 RFLP, 25 AFLP, 3 morphological, 1 biochemical markers	12	972 cM	0.18	Menéndez et al. (1997)
V. unguiculata (IT84S-2049) × *V. unguiculata* (524B)	Recombinant inbred (94)	133 RAPD, 36 RFLP, 267 AFLP, 3 morphological, 1 biochemical markers	11	2,670 cM	0.197	Ouédraogo et al. (2002a)
V. vexillata var. *angustifolia* (Tvnu 1443) × *V. vexillata* var. *vexillata* (Tvnu 73)	F_2 (94)	70 RAPDs, 47 AFLP, 1 SSR, 2 morphological traits	14	1,564.1 cM	0.335	Ogundiwin et al. (2005)
V. angularis (Tanba Dainagon) × *V. riukiuensis*	BC_1F_1 population (77)	100 SSR, 47 RFLP, 234 AFLP markers	11	624 cM	0.029	Kaga et al. (2003)
V. nepalensis (JP107881) × *V. angularis* (JP81481)	BC_1F_1 (187)	205 SSR, 94 RFLP, 187 AFLP markers	11	832 cM	0.039	Han et al. (2005)
V. nepalensis (JP107881) × *V. angularis* (JP81481)	F_2 (141)	74 SSR markers	11	650 cM	0.280	Isemura et al. (2007a)
V. angularis var. *angularis* (JP109685 cv. Kyoto Dainagon) × *V. angularis* var. *nipponensis* (JP110658)	F_2 (188)	191 SSR, 2 STS, 1 CAPS, 2 SCAR, 36 AFLP markers	10	772 cM	0.039	Kaga et al. (2008)
V. mungo (JP219132) × *V. mungo var. silvestris* (JP107873)	BC_1F_1 population (180)	61 SSR, 59 RFLP, 27 AFLP, 1 morphological markers	11	783 cM	0.000	Chaitieng et al. (2006)

V. mungo (*TU94-2*) × *V. mungo* var. *silvestris*	F_9 RI population (104)	47 SSR, 254 AFLP, 86 RAPD, 41 ISSR	11	865.1 cM	0.44	Gupta et al. (2008)
V. umbellata (JP100304) × *V. nakashimae* (JP107879)	F_2 (74)	101 SSR, 74 RFLP markers	11	652 cM	0.260	Somta et al. (2006)
V. umbellata (cultivated, JP217439) × *V. umbellata* (wild, JP210639)	BC_1F_1 (198)	185 SSR, 103 AFLP markers	11	814 cM	0.192	Isemura et al. (2007b)
V. radiata (cultivated, cv. Sukhothai) × *V. radiata* (wild, W166)	BC_1F_1 population (250)	153 SSR markers	11	674 cM	0.137	Isemura et al. (2008)

[a]Linkage groups resolved

people (Lawn and Cottrell 1988). It is a useful legume to help prevent soil erosion due to its fast growth. A domesticated form of *V. vexillata* has been reported from Bali, West Timor, and Indonesia, where it is used for seeds and tubers (Karuniawan et al. 2006). *V. vexillata* has a number of traits that would be useful for cowpea breeding, but it is cross-incompatible with cowpea. An approach to introducing genes from *V. vexillata* into cowpea would be to identify genome regions with useful genes, clone them, and put them in cowpea by genetic transformation. To this end, a genome map of *V. vexillata* has been constructed (Ogundiwin et al. 2005). The resulting genome map had 70 RAPD markers, 47 AFLP markers, and one SSR marker mapped to 14 linkage groups compared to the expected 11, the haploid chromosome number. This linkage map enabled positioning of various QTLs including that for cowpea mottle carmovirus CPMoV resistance (Ogundiwin et al. 2005).

15.7.5 Mungbean

It is only recently that a genetic map of mungbean with the expected 11 linkage groups of this species has been reported (Isemura et al. 2008; Table 15.3). A comparison across the genus *Vigna* with regard to a QTL related to the important domestication-related trait seed size is shown in Fig. 15.6.

15.7.6 Rice Bean

Rice bean is not a major cultivated legume; however, it is locally important in parts for South and Southeast Asia. It produces profuse numbers of pods. Its major interest to Asian *Vigna* specialists is its useful source of resistance to pests and diseases. Resistance to mungbean mosaic virus, one of the most devastating diseases of this crop, has been found in rice bean (Pandiyan personal communication). It is also a source of bruchid resistance (Tomooka et al. 2000). Consequently a genetic map of this species has been developed (Isemura et al. 2007c). Domestication-related traits have also been analyzed in a cross between cultivated and wild rice bean (Isemura et al. 2007b)

15.8 Genomic Resources and Collaborative Initiatives

An increasing number of genome resources are now available for *Vigna*. The National Institute of Agrobiological Sciences (NIAS) has developed SSR markers for azuki bean that have been used successfully in mapping other Asian *Vigna* (Wang et al. 2002; Chaitieng et al. 2006; Gupta et al. 2008; Somta et al. 2008b), and primer information is available at: (*http://www.gene.affrc.go.jp/databases-marker_information_en.php*).

Using genome arrays from soybean enabled many single feature polymorphisms to be detected in cowpea (Das et al. 2008). Genomic sequences of the hypomethylated portion of the cowpea genome have been reported (Chen et al. 2007; Timko et al. 2008). Bacterial artificial chromosome (BAC) libraries of three cowpea genotypes are leading to the finding of new markers, such as 9,077 SSRs and SNPs, genomic amplicons of conserved orthologous sequences and ESTs (Close 2008). The NordEST network is developing ESTs and SAGE tags for cowpea (Close 2008).

Support, particularly for research on cowpea genomic resources and cowpea improvement, is coming from the CGIAR Generation Challenge program (*http://www.generationcp.org/index.php*), USAID-CRSP (http://crsps.org/), Gates Foundation (*http://www.entm.purdue.edu/NGICA/*), and the Kirkhouse Trust, among others. This is largely in response to the challenges of increasing food security in sub-Saharan Africa.

The various *Vigna* genomic initiatives will undoubtedly be used to understand not just the crops themselves but also the gene pools including wild and weedy species of which they are a part.

15.9 Future Perspectives

This review has discussed the considerable research that has been conducted on the wild relatives of crops in the genus *Vigna*. However, there are major gaps in our knowledge of these genetic resources, and the research on *Vigna* as a whole is poorly coordinated as it lacks the cooperative networks that exist for other crops. Particularly, as advances are made in genome mapping and genomics of *Vigna*, enhanced

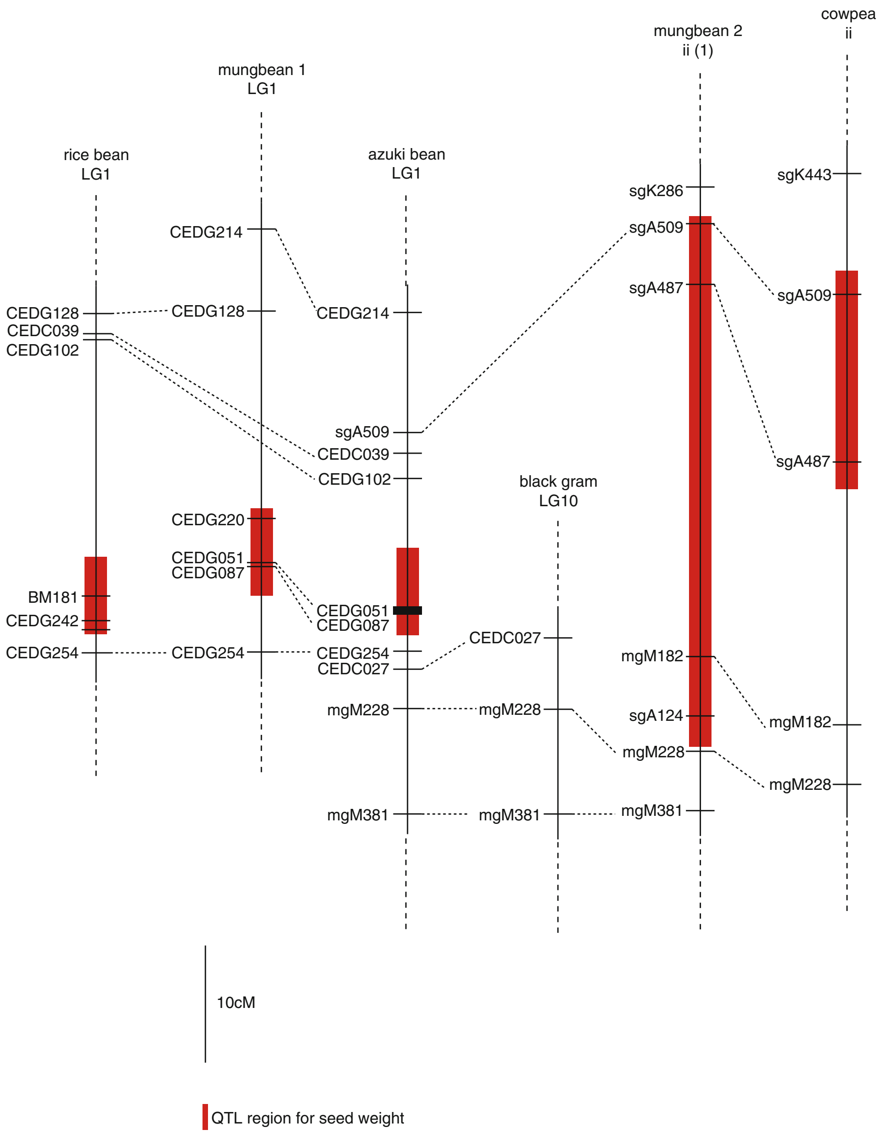


Fig. 15.6 A major QTL for seed weight on linkage group 1 of azuki bean compared with QTL for seed weight at a similar genomic position in other *Vigna* species. Of *Vigna* species analyzed to date only black gram did not have a QTL for seed size at a similar location to azuki bean. References: Rice bean (Isemura et al. 2007b), mungbean 1 (Isemura et al. 2008), mungbean 2 (Fatokun et al. 1992, Menacio-Hautea et al. 1993), azuki bean (Isemura et al. 2007a), black gram (Chaitieng et al. 2006), cowpea (Fatokun et al. 1992)

collaboration will be necessary to ensure some standardization for such things as gene symbols, molecular markers, and linkage groups.

There are also significant gaps in the germplasm collection for wild *Vigna*. This is particularly true for species of Latin America (subgenus *Sigmoidotropis*). Future research needs to focus on ways to transfer useful genes from one species to another.

15.10 Databases that Include Data Related to *Vigna* Species

http://www.nenno.it/Beanref/

BeanRef is a collection of external links and references from literature to different aspects of research on beans (*Phaseolus* and *Vigna*).

http://beangenes.cws.ndsu.nodak.edu/

A *Phaseolus*/*Vigna* database.

http://www.kew.org/herbarium/legumes/beanbag.html

Bean Bag is a newsletter to promote communication among research scientists concerned with the systematics of the Leguminoseae/Fabaceae.

http://www.grainlegumes.com/

A grain legumes portal that provides abundant information on conferences, research, and publications related to grain legumes, particularly from a Eurpoean perspective.

http://www.br.fgov.be/RESEARCH/COLLECTIONS/LIVING/PHASEOLUS/index.html.

This database provides information on Wild Phaseoleae – Phaseolinae Collection of the National Botanic Garden, Belgium.

http://www.gene.affrc.go.jp/publications.php?type=report&section=plant

Germplasm collection reports in English that have focused on Asian *Vigna* collection by the *Vigna* group at NIAS can be found at this web site.

http://www.gene.affrc.go.jp/databases-marker_information_en.php

Information related to SSR markers used in the genome maps developed by the *Vigna* group at NIAS and links to some of their publications can be found at this website.

http://www.becabioinfo.org/bbrweb/cms/iita-bioinformatics/CowpeaSNP/putative_cos.php

The Cowpea SNP project with information on primers associated with various potentially useful genes can be found at this website.

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